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THE RELATION BETWEEN THE DISTRIBUTION OF IRON AND ASCORBIC ACID IN THE BODY.*

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EARLY in the course of a study on the ultimate fate and delayed effects of intravenously injected saccharated iron oxide, the selective accumulation of iron in the suprarenal cortex was noted (Nissim, 1949). Ascorbic acid is present in the suprarenal cortex in particular abundance, and the idea that the iron found its way to the suprarenal cortex because of some metabolic association in the body between the two substances appeared plausible and worthy of investigation. This idea was particularly supported by the great affinity which was observed between iron and ascorbic acid *in vitro* during the work on the preparation of "ferric hydroxide ferrous ascorbate."

The distribution of iron in the epithelial cells of the suprarenal cortex had been studied in detail in the mouse, and, as noted previously (Nissim, 1949), it followed a well-marked pattern, with the heaviest deposit in the zona reticularis and inner fasciculata. This faded in the outer fasciculata, but usually became heavier again in the glomerulosa. The first task was therefore to see whether the distribution of ascorbic acid in the suprarenal cortex actually corresponded to the same pattern. Study of previous publications showed that various authors agreed in finding the greatest concentration of ascorbic acid in the reticularis and inner fasciculata, but there was a similar general agreement that it was absent from the zona glomerulosa (Bourne, 1933, 1934; Giroud and Leblond, 1935; Leblond and Gardner, 1938; Westergaard, 1934). Several species of animals were used by these workers, but Leblond and Gardner, who made a special study of the distribution of ascorbic acid in the mouse, stated that "none was observed in the glomerular zone." It seemed useless, therefore, to follow this matter any further.

* Abridged from Chapter XV, vol. 3, of M.D. Thesis entitled "Intravenous Iron," University of London, 1949.

More recently, however, Deane and Morse (1948) published their findings on the distribution of ascorbic acid in the suprarenal cortex of the rat. These workers claimed that ascorbic acid was present in most of the cells of the zona glomerulosa in normal animals, and that previous investigations failed to demonstrate it in this zone because of "less satisfactory procedures for the preservation of the acid." Interest in the possible correspondence of the distribution of iron and of ascorbic acid was stimulated afresh, and this matter was studied using the staining technique of these workers. Owing to the smaller size of the suprarenal glands in mice, the times taken for the staining with silver nitrate and fixation in hypo solution were cut short. The prolonged procedure of Morse (1945) for the subsequent dehydration of the glands was dispensed with and short changes in absolute alcohol were used instead. Ten per cent silver nitrate solution was also used instead of the saturated solution of Barnett and Bourne (1940). Filtering the silver nitrate solution with a Whatman No. 50 filter-paper in the dark immediately before use was found to reduce the occurrence of artefacts. The modified method is described below.

METHODS.

The mice were killed by neck stretching, and both adrenals rapidly removed and immersed in a freshly prepared and filtered silver nitrate fixing solution contained

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DESCRIPTION OF PLATES.

FIG. 1.—Suprarenal of mouse showing the zonal distribution of iron in the cortex of the gland. Animal received a total of 2·16 g. Fe/kg. as saccharated iron oxide in 12 weekly intravenous injections, and died about three months after the last injection. Prussian blue and carmalum. × 100.

FIG. 2.—Suprarenal of normal mouse showing distribution of ascorbic acid in zona glomerulosa in relation to covering layer of fat. Acid silver nitrate and eosin. × 370. Note silver granules receding from edge where covering fat is absent.

FIG. 3.—Suprarenal of normal mouse showing distribution of ascorbic acid after exposing tissue to reducing effect of H_2S. Acid silver nitrate and eosin. × 370. Note silver granules in zona glomerulosa right up to edge irrespective of covering fat.

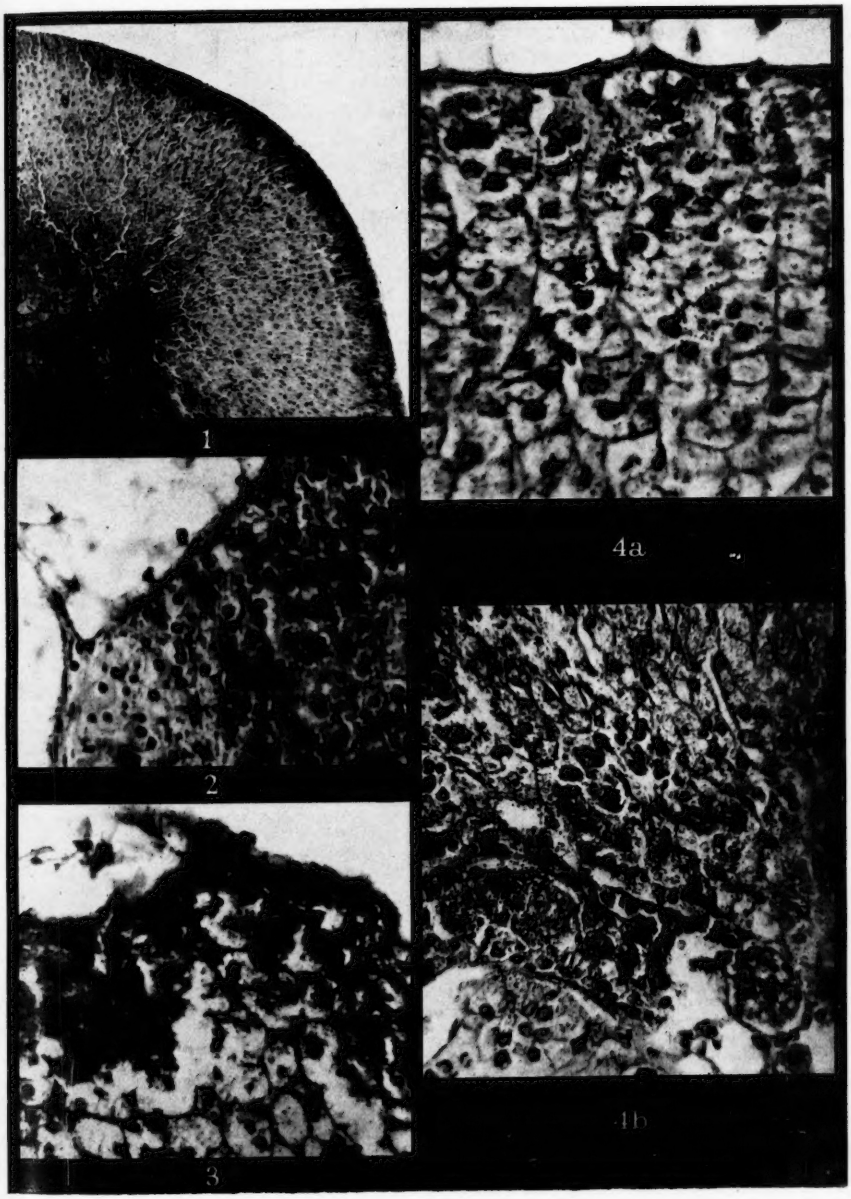
FIG. 4.—Suprarenal of normal mouse showing the usual relative concentration of ascorbic acid in the three cortical layers of the gland. Acid silver nitrate and eosin. A. Zona glomerulosa and outer fasciculata. × 450. B. Zona reticularis and inner fasciculata. × 325. Compare distribution of iron in Fig. 2.

FIG. 5.—Suprarenal of mouse showing heavier iron staining in zona glomerulosa than in outer fasciculata irrespective of covering fat. Animal received a total of 1·26 g. Fe/kg. as saccharated iron oxide in 7 weekly intravenous injections, and was killed 4 months after the last injection. Prussian blue and carmalum. × 190.

FIG. 6.—Suprarenal of normal rat showing distribution of ascorbic acid in outer layers of cortex. Acid silver nitrate and eosin. × 500. Note heavier deposit of granules in junctional zone of glomerulosa and outer fasciculata, and in capsule. Heavy silver deposit in reticularis and inner fasciculata is not shown in figure.

FIG. 7.—Suprarenal of rat showing distribution of iron in cortical layers of the gland. Animal received a total of 1·0 g. Fe/kg. as saccharated iron oxide in a series of 24 weekly injections, and was killed 2 weeks after the last injection. Prussian blue and carmalum. × 90. Note three bands of heavy iron staining.

FIG. 8.—Ovary of normal mouse showing distribution of ascorbic acid in the various elements. Acid silver nitrate and eosin. × 300. Silver granules are absent from zona granulosa of Graafian follicles, but present in ovum at one side of outer rim, and in interstitial tissue outside follicle.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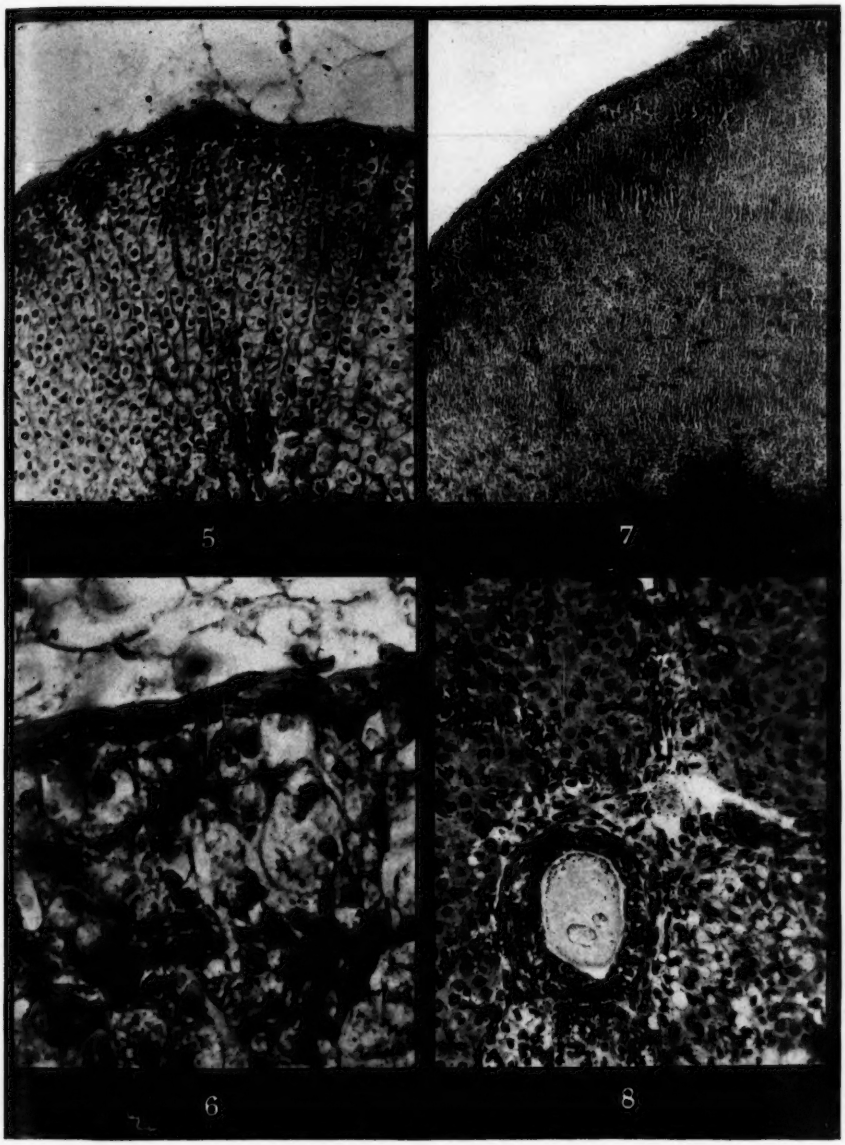

Nissim.

in a light-tight small bottle. The silver nitrate fixing solution was prepared as follows:

Ethyl alcohol	50 ml.	pH approximately 2·0.
Distilled water	40 ml.	
Glacial acetic acid	10 ml.	
Silver nitrate (fresh and kept in dark)	10 g.	

The glands were left in this solution for 15–20 min. and then transferred with paraffinised forceps to the following acidified hypo solution, in which they were left for 1 hr.:

Sodium thiosulphate	5·0 g.	pH approximately 5·0.
Sodium bisulphite	1·0 g.	
Distilled water	100·0 ml.	

They were then removed with paraffinised forceps, washed in several changes of tap-water, dehydrated in three changes of absolute alcohol at intervals of ½ hr., cleared in carbon bisulphide and chloroform for about 10 min., and embedded in paraffin. Sections were counterstained with either carmalum or haematoxylin and eosin.

RESULTS.

The Distribution of Ascorbic Acid and of Iron in the Suprarenal Cortex of the Mouse.

The suprarenal glands of several mice were stained by the above technique. The silver granules were heaviest in the zona reticularis and inner fasciculata. The ascorbic acid was mostly absent from the glomerulosa, but in a few sections the glomerulosa showed heavy deposits of silver over small parts of the circumference, thus giving in those areas a distribution corresponding exactly to that of iron (Fig. 1). It was noted in one section which showed this heavy silver deposit in the glomerulosa that the particular part of the circumference was covered with fat. The ascorbic acid reached the surface wherever the capsule was covered with fat and receded from the glomerulosa just where the fat stopped (Fig. 2). This gave a clue which was profitably followed up in the experiments related below.

Relation of ascorbic acid to surrounding fat.

The relation of the covering fat to the ascorbic acid of the glomerulosa in the mouse was shown to the best advantage in serial sections of suprarenal glands taken from a number of fat as well as lean mice. Care was taken to remove the glands with all their surrounding fat. In lean mice which had no surrounding fat no ascorbic acid could be found in the glomerulosa, but in fat mice the distribution of the silver granules in this zone depended on the exact plane of section. The peritoneal surface of the glands was always free of fat, and therefore always devoid of silver granules in the glomerulosa. In serial sections cut at different planes it could be shown that the distribution of ascorbic acid in this outer zone was invariably related to the fat covering, and in sections cut below the plane of the peritoneal surface a complete ring of ascorbic acid in the glomerulosa could be demonstrated.

The question arose whether there was a real absence of ascorbic acid from the zona glomerulosa in the part not covered by fat, or whether the ascorbic acid was present but in a form which could not reduce the silver nitrate, i.e., in the oxidised form. The latter of the two alternatives appeared the more likely. It was thought possible that the ascorbic acid in the thin superficial layer of the exposed parts of the glands may be rapidly oxidised in the air in the short time between the opening of the abdomen and the immersion of the suprarenals in the silver nitrate solution. To prevent this happening the following experiment was performed :

Mice were killed and immediately injected with the silver nitrate fixing solution intraperitoneally. A glass needle was used, and this was connected to an all-glass syringe with thin rubber tubing. After the peritoneal cavity was filled with the silver nitrate solution the injection puncture was clamped with artery forceps, and the animal kept in the dark for 20 min. The suprarenals were then removed and placed in hypo, and the procedure completed as before. Serial sections of suprarenals treated in this way still showed the same relation of ascorbic acid to fat in the glomerulosa. It was concluded that if ascorbic acid was in fact present in the glomerulosa in the parts not covered by fat, it would be present in the oxidised state in the live animal.

The Bourne silver stain for oxidised ascorbic acid, as described by Glick (1949) was used on two mice. The fresh tissue was exposed to the vapour of glacial acetic acid for several minutes, then placed in an atmosphere of H_2S for 15 min. in order to reduce the oxidised ascorbic acid. The H_2S was then removed by placing the tissue in vacuum for 30 min., then in a stream of nitrogen for 15 min. The tissue was then treated with silver nitrate as before. Serial sections of the suprarenal glands treated in this way exhibited a complete ring of silver granules in the glomerulosa irrespective of relation to fat (Fig. 3). Such sections showed complete correspondence between the distribution of ascorbic acid and iron in the suprarenal cortex of the mouse (Fig. 4).

Distribution of iron in relation to the covering fat.

Three mice were given seven weekly injections of 180 mg. Fe/kg. as saccharated iron oxide, totalling 1·26 g. Fe/kg. Three months from the last injection the animals were killed, their suprarenals removed with their surrounding fat, and serial sections cut. Such sections showed the epithelial cells of the glomerulosa laden with iron irrespective of the covering fat (Fig. 5). Although the iron deposit was heavier in the glomerulosa than in the outer fasciculata in most of the mice, this was not so in all. This again corresponded with the state of affairs regarding the relative concentration of ascorbic acid in this zone.

Distribution of ascorbic acid in the rat suprarenal.

The distribution of ascorbic acid and of iron was next studied in the suprarenal cortex of the rat. Rat suprarenals stained for ascorbic acid with silver nitrate showed a distribution of silver granules different from that in the mouse and corresponding exactly to that described by Yoffey (1949). The concentration was heaviest in the zona reticularis, but became heavy again in the outer fasciculata just bordering on the glomerulosa. The zona glomerulosa itself was not free from ascorbic acid but the granules diminished appreciably. The silver granules

became heavy again in the fibrous capsule (Fig. 6). It was interesting to find that in the rat as well this granular staining was absent from the glomerulosa and the fibrous capsule in the areas not covered by fat, and here again this must be due to the presence of ascorbic acid in the oxidised form.

Distribution of iron in rat suprarenal cortex.

Rats were given repeated intravenous injections of 100 mg. Fe/kg. as saccharated iron oxide at 7–14 days' intervals until a total dose of 0·5–1·0 g. Fe/kg. was reached. They were killed 14–30 days after the last injection. Serial sections of suprarenals of these rats showed an iron distribution in the cortex which corresponded exactly to that of ascorbic acid. There were thus three heavy bands of both ascorbic acid and iron in the suprarenal cortex of the rat, the zona reticularis, the junctional zone of fasciculata and glomerulosa, and the fibrous capsule of the gland, with fainter staining in between (Fig. 7). The distribution of iron in the outer zones, as in the mouse, bore no relation to that of the fat covering.

The Distribution of Ascorbic Acid and of Iron in the Ovaries and in the Fibrous Tissue of Healing Wounds.

The correlation in the distribution of ascorbic acid and of iron already established for two different species in the suprarenal cortex was followed up further. Ascorbic acid is known to occur in the highest concentrations in the adrenal cortex, the ovary—especially in the corpora lutea, the anterior pituitary, and the interstitial tissue of the testis (Biskind and Glick, 1935 ; Giroud and Leblond, 1935). It also occurs in high concentrations in collagenous fibrous tissue, and especially in young granulation tissue during the process of wound repair (Lauber and Rosenfeld, 1938). In the liver it occurs in fair concentrations. As the distribution of iron in the ovaries had been already studied in detail (Nissim, 1949), it was only necessary to carry out silver staining experiments for ascorbic acid in these organs to see if the correspondence with iron distribution would hold here as well.

Distribution of ascorbic acid in the mouse ovary.

Female white mice were killed and their ovaries stained with silver nitrate. Ascorbic acid was present in the germinal epithelium, in the interstitial cells of the ovaries, and in the interspersed luteal cells and histiocytes (Fig. 8). The most striking feature was the extreme scarcity or total absence of granules in the zona glomerulosa of the Graafian follicles. The cytoplasm of a large proportion of ova showed a fairly heavy deposit of silver granules. This was often most marked in the region of the zona pellucida. Some ova, however, were free from ascorbic acid. This description corresponds very closely to that given for rats by Hoch-Ligeti and Bourne (1948).

It is seen that the correspondence between the distribution of iron and ascorbic acid holds in the case of the ovaries as well. The only discrepancy seems to be between the proportion of ova which show iron and those which show ascorbic acid in their cytoplasm, that of the latter being much higher. This difference may be explainable, however, by the fact that this particular site is concerned more with the storage than with the metabolism of ascorbic acid. If the iron were linked with the metabolism of ascorbic acid in some way, the deposit of iron would only

become demonstrable if a high metabolic turnover of the vitamin exists. In unfertilised ova the metabolic turnover is likely to be low although the actual content of the various essential nutritive metabolites is high.

Distribution of iron in healing wounds.

For the demonstration of iron in healing tissue the following experiment was performed : A rabbit was given a first injection of saccharated iron oxide after a laparotomy, followed by 7 further daily intravenous injections of the same dose (45 mg. Fe/kg.) A fortnight after the last injection the laparotomy wound was dark brown in colour. The wound was excised and sections of it were stained for iron as well as with haematoxylin and eosin. There was a particularly abundant infiltration with histiocytes, and these cells stood out by reason of their massive iron impregnation. In addition, the young fibrocytes and the wavy bands of the newly formed collagen exhibited a particularly vivid Prussian blue staining. The correlation between iron and ascorbic acid distribution thus appears to be consistent, and one which merits further study.

DISCUSSION.

Perhaps the most striking part of this evidence here presented relating iron and ascorbic acid in the tissues investigated is their identical distribution in the suprarenal cortex of both the mouse and the rat, a distribution which presents markedly individual features in the two species. The question arises, however, whether this correspondence is due to a direct relationship or due to an associated or even independent third factor which also has the same distribution as ascorbic acid. At present it is impossible to answer this question with certainty, but a number of points which bear on this problem will be discussed.

Steroids and iron.

The presence of ascorbic acid in the suprarenal cortex has always been associated by various workers with the capacity of the constituent cells to produce steroid hormones. It may be asked, therefore, whether the deposition of iron in the cortical cell may be associated with the steroids rather than with ascorbic acid itself. Two facts appear to favour the former of the two hypotheses. Firstly, there is no known affinity between iron and the steroid hormones as there is in the case of ascorbic acid. Neither cholesterol nor whole adrenal extract exhibited any reactivity with iron in the test-tube under varying conditions. Secondly, the distribution of the steroids seems to be slightly different from that of either ascorbic acid or iron. According to Deane and Morse (1948), the maximum concentration appears to be in the glomerulosa and reticularis. The maximum concentration of ascorbic acid, on the other hand, is in the transitional zone between the glomerulosa and outer fasciculata, inner fasciculata and reticularis. The possibility should not be denied, however, that the sites of maximum productivity of the cortical hormones may not correspond to those of maximum storage, and this question must therefore remain for the time being an open one.

Relation between ascorbic acid and iron metabolism.

The evidence advanced by various authors on the relationship between ascorbic acid on the one hand and iron metabolism and erythropoiesis on the

other has been extremely conflicting. The results obtained by earlier workers (Schröder and Braun-Stappenbeck, 1941 ; Albers, 1942 ; Videback and Alstead, 1943) suggested some relationship between ascorbic acid and iron metabolism in the body, but more recent reports (Neuweilier, 1945, 1948 ; Tötterman, 1949) seem to be in direct contradiction. The work of Tötterman shows that there is no correlation between serum ascorbic acid and serum iron, blood haemoglobin, or erythrocytes. This author also showed that ascorbic acid was without effect on the anaemia of infection.

The findings of Tötterman, however, constitute no evidence against ascorbic acid playing some important rôle in iron metabolism. Ascorbic acid may not hasten the removal of iron from the blood, but if ascorbic acid does combine with iron in the tissues, the resulting iron complex may be very diffusible and analogous to " ferric hydroxide ferrous ascorbate " (Nissim, 1949). Such a diffusible iron complex need not produce a detectable rise in serum iron in a slow mobilisation process. A study which might yield helpful results under such circumstances would be one of the direct effect of ascorbic acid on urinary iron excretion. A further point which deserves mention is that if ascorbic acid does reduce the iron in the body, it would itself become oxidised to dehydroascorbic acid and perhaps even to diketogulonic acid. For proper correlation studies between ascorbic acid and iron, it is felt that the oxidation products of ascorbic acid should be taken into account. The usual methods for ascorbic acid estimation such as those used by Tötterman would be insufficient, as they merely estimate reduced ascorbic acid. By contrast, the method of Roe and Kuether (1943), or some modification of it (Glick, 1949), estimates both dehydroascorbic acid and diketogulonic acid as well as reduced ascorbic acid. Such estimations would perhaps give a more accurate picture of the relationship between the two substances.

Finally it is suggested that much may still be learnt from the work of Moore, Bierman and Minnich (1940) on the relation between ascorbic acid and iron in the body. These authors studied the effect of large doses of iron on the ascorbic acid levels of the serum, an aspect the reverse to that which engaged Tötterman's attention. Moore *et al.* showed that intravenous injection of highly ionised simple ferrous or ferric salts caused a temporary total disappearance of titrable vitamin C from the plasma. They further showed that the plasma contained a protective substance which normally protected against the oxidation of the ascorbic acid it contains, e.g., by smaller doses of iron. The parenteral administration of large amounts of ascorbic acid did not, however, hasten the rate at which the iron was removed from the blood.

These results may offer an explanation for Tötterman's failure to show a relationship between ascorbic acid and iron, and at the same time they advance reasonable evidence for a link between these two substances *in vivo*.

Other physiological substances and iron.

The number of physiological substances that take part in the absorption, transport, excretion and storage of iron is unknown. Although the present work suggests that ascorbic acid is directly involved in this distribution, at least under the conditions of the experiments, abundant evidence is accumulating regarding the rôles played by a number of other physiological iron compounds or complexes. Some of these must also be directly concerned in this distribution. Thus, in experimental siderosis there may be an increase in ferritin in the liver, spleen,

kidneys and intestinal epithelium. Ferritin itself does not stain with Prussian blue, and none of the iron accumulations described here and in a previous paper (Nissim, 1949) can be directly due to it, though " haemosiderin " may have become heaped up on a foundation of this protein complex. No remaining brown pigment, however, was detected in sections stained lightly with Prussian blue.

Para-aminobenzoic acid, lactic acid, pyruvic acid, adenylic acid, the amino acids as well as other physiological substances with affinity for iron may also have to be considered in the study of iron distribution in siderosis.

" Ferric hydroxide ferrous ascorbate " and the suprarenal cortex in the guinea-pig.

It was shown previously (Nissim, 1949) that when " ferric hydroxide ferrous ascorbate " was injected into guinea-pigs almost no iron appeared in the suprarenal cortex. This was in contrast to the fairly heavy iron storage which was obtained with saccharated iron oxide in only one-tenth of the dosage of " ferric hydroxide ferrous ascorbate." Indeed, the original reason for using this latter preparation was that this form of iron was expected to appear in far greater concentrations in the suprarenal cortex, on account of its " ascorbate " radicle. The exact opposite resulted. It therefore seems that iron already linked to " ascorbic acid " is capable of passing through the suprarenal cortex without being fixed by the free ascorbic acid which is there. Nevertheless, there may be other less obvious factors involved. It would consequently be interesting to find out if other *diffusible* iron preparations would penetrate into these cells of the suprarenal cortex. From the work of Rothlin (1944), however, who gave a number of ferrous iron compounds to a variety of animals, including guinea-pigs, it appears that definite iron deposits would still accumulate in the cortex of the gland. These experiments seem therefore to lend support to the conception of some metabolic linkage between iron and ascorbic acid, at least in certain parts of the body. It should be pointed out, however, that Polson (1933), using dialysed iron (B.D.H.) in rabbits, found diffuse and granular iron in the endocrine cells of the medulla and none in the cortex. Indeed, the whole problem may be far more complex than appears at first sight.

SUMMARY.

The heavy accumulation of iron in the suprarenal cortex of mice after saccharated iron oxide administration was found to correspond exactly with that of ascorbic acid except that the latter was absent in the superficial areas of the suprarenal cortex which were not covered with fat. Reduction with hydrogen sulphide before staining with silver nitrate showed that ascorbic acid was present in these areas in the oxidised form and the correspondence between iron and ascorbic acid distribution thus became complete.

In the rat the distribution of ascorbic acid is different from that in the mouse, and the distribution of the iron in this species again follows a corresponding pattern.

There is a close similarity in the distribution of both substances in the ovaries and in young fibrous tissue.

Iron is not deposited in the suprarenals of animals receiving an iron ascorbate preparation, possibly because iron already joined to an ascorbate radicle fails to be fixed by the epithelial cells.

The possible relationship between iron and ascorbic acid as well as other physiological substances in the body is discussed.

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ELECTROPHORETIC STUDIES OF VIRUS-RED CELL INTERACTION: RELATIONSHIP BETWEEN AGGLUTINABILITY AND ELECTROPHORETIC MOBILITY.*

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PREVIOUS papers in this series (Ada and Stone, 1950; Stone and Ada, 1950) have been concerned with the reduction of electrophoretic mobility which occurs when human red cells are treated with viruses of the mumps-influenza group. It was found that strains varied in the extent to which they could reduce electrophoretic mobility, and the series formed by arranging them in order of this capacity closely resembled the "receptor gradient" (Burnet, McCrea and Stone, 1946), based on the progressive loss of agglutinability of erythrocytes by these viruses.

Two exceptions were encountered, namely the viruses of swine influenza and of Newcastle disease of fowls, which reduced electrophoretic mobility further than expected from their position in the receptor gradient. It was suggested that these strains had an additional action on the red cell, which lowered the mobility but had little effect on agglutinability. The "receptor-destroying enzyme" of *V. cholerae* possessed both types of activity, and also reduced the electrophoretic mobility further than any of the viruses.

To obtain more precise information on the cellular receptor we approached the problem by two methods. First the *course* of virus action was studied in terms of changes in agglutinability and electrophoretic mobility. Secondly, controlled *partial treatments* with one virus were followed by complete action of another. The sum of the two actions was compared with the complete action of the second agent only and the differences, if any, examined in the light of our working hypothesis.

MATERIALS AND METHODS.

Reagents.

Normal saline and calcium acetate saline were prepared as described by Ada and Stone (1950).

Viruses.

MU: Mumps virus, obtained through the courtesy of Dr. J. F. Enders.
NDV: Newcastle disease virus of fowls, Victorian strain of Albiston and Gorrie (1942).
MEL: Melbourne strain of influenza A (Burnet, 1935).
PR8: Influenza A (Francis, 1934).
BEL: Influenza A, isolated from Victorian epidemic (Burnet, Beveridge, Bull and Clark, 1942).

* This work was aided by a grant from the National Health and Medical Research Council, Canberra, Australia.

LEE : Influenza B (Francis, 1940).
SW : Shope's strain 15 of swine influenza (Shope, 1931).
MIL : Influenza B, isolated from Melbourne epidemic (Burnet, Stone and Anderson, 1946).

Stocks were grown in the allantoic cavity of fertile hens' eggs. Mumps virus was inoculated into 8-day eggs and the allantoic fluid harvested after incubation for five days. All other strains were inoculated into 11-day eggs, the fluids being harvested after 42 hr. incubation. For treatment of red cells MIL virus was used as an eluate prepared by adsorption to and elution from fowl red cells.

Purified mumps (MU_p) and purified Newcastle disease (NDV_p) viruses were prepared by high speed centrifugation (25,000 g. for 75 min. at room temperature). The deposit was re-suspended in normal saline, one-tenth of the original volume.

RDE ("receptor destroying enzyme") of V. cholerae.

Crude extracts were obtained by the method of Burnet and Stone (1947). These were purified and concentrated according to Ada and French (1950).

Red blood cells.

Washed human group O cells from individual donors were used within 48 hr. of collection. It was found in preliminary tests that cells from different donors did not differ in their reactions.

Stabilization of virus-treated red cells.

Twofold dilutions of antiserum in 0·25 ml. volumes of normal saline were mixed with 0·25 ml. of a 1 per cent suspension of the virus-treated cells. After thorough shaking the cells were allowed to settle at room temperature. The smallest amount of serum inhibiting agglutination was determined by observing the pattern of sedimented cells. Serum was added in the same relative amount to a 5 per cent suspension of the treated cells.

Test of red cells for agglutinability.

Equal volumes (0·25 ml.) of a 1 per cent suspension of stabilized red cells were mixed with dilutions of the standard series of test-viruses, containing exactly 5 agglutinating doses. The degree of agglutination was read by the pattern of red cells after settling at room temperature. One agglutinating dose is the amount of virus capable of causing partial ("+") agglutination of 0·25 ml. of 1 per cent normal human red cells at room temperature.

Microelectrophoresis.

Electrophoresis was carried out in a M/15 sodium potassium phosphate buffer (Clark, 1920) to which 1 per cent (w/v) glucose had been added; pH $7{\cdot}35 \pm 0{\cdot}05$. Cells to be examined were washed once in the buffer before re-suspension for testing. The microelectrophoresis apparatus was designed and largely constructed by Mr. H. F. Holden; the basic measurements of the cell itself were within the range given by Abramson, Moyer and Gorin (1942). Before each series of tests the cell was coated with a 1 per cent solution of gelatine in water (Miller, Lauffer and Stanley, 1944), ensuring a symmetrical depth-velocity parabola. The electrophoretic cell was held in a small bath through which was circulated water at $25 \pm 1°$. In practice a minimum of 10 readings was made at the 2 theoretical levels and the results averaged. After measurement of each sample the cell was thoroughly washed through with buffer before the introduction of the next sample. Control tests with normal erythrocytes were carried out before and after each set of estimations.

Calculation of agglutinability endpoints.

The agglutinability endpoint for a certain strain of virus is that electrophoretic mobility value at which 5 agglutinating doses of this virus give partial "+" agglutination. By definition, this level corresponds to an 80 per cent reduction in agglutinability for the respective strain.

In any single experiment this endpoint can be expressed as a range of electrophoretic mobility values within which the "+" reaction would fall. These ranges were read as the distance between the $\pm$ and the $\pm\pm$ degrees of agglutination. In computing the average

from several experiments, it appeared advisable to weight the individual results so that those from a narrow range contributed more to the average than those from a wide range. The weighting factors were as follows:

Mobility range.	Weighting factor.
<0·025	12
0·025–0·05	6
0·05 –0·10	3
0·10 –0·20	1·5
>0·20	1

EXPERIMENTS.

Changes in Agglutinability and Electrophoretic Mobility in the Course of Receptor Destruction.

In studying the course of viral action on the red cell a comparison was made of viruses supposed to have only a single type of action with those agents (SW, NDV and RDE) which were thought to have an additional action. Preliminary tests confirmed our assumption based on earlier work that for all agents in the first group the relationship of their effect on agglutinability and electrophoretic mobility was closely similar. The detailed investigations, therefore, could be restricted to a few representative strains only; MEL, LEE and MIL were chosen.

The different stages in the course of an enzyme action can be studied either by using a standard amount of enzyme and substrate and taking samples at intervals, or by keeping constant the amount of substrate and the time of interaction and varying the amount of enzyme. To avoid errors incurred in stopping the reaction after very short periods the latter method was used where practicable.

The virus preparations were diluted in twofold steps in normal saline. RDE dilutions were made similarly in calcium acetate saline. A series of fourteen steps was found more than sufficient in every instance to cover the range from complete to undetectable action. To each tube was added 0·25 ml. of packed human red cells, and the mixtures incubated at 37° for periods which for the starting dilution of each particular agent had previously been shown to result in maximal action on red cells. At the end of the incubation period the cells were spun

TABLE I.—*Agglutinability Endpoints during the Course of MEL, LEE and MIL Virus Action.*

Agent.	Agglutinability endpoint* (weighted mean ± standard error) for							
	MU.	MU_p.	NDV.	NDV_p.	MEL.	PR8.	BEL.	LEE.
MEL (2)†	1·19 ± 0·10	1·10 ± 0·10	1·05 ± 0·08	0·87 ± 0·05	0·96 ± 0·06	0·92 ± 0·10	— —	— —
LEE (4)	1·02 ± 0·03	0·98 ± 0·01	1·01 ± 0·03	0·87 ± 0·04	1·01 ± 0·03	0·93 ± 0·01	0·82 ± 0·01	0·72 ± 0·02
MIL (4)	1·06 ± 0·05	0·96 ± 0·01	0·98 ± 0·01	0·82 ± 0·02	0·93 ± 0·04	0·83 ± 0·04	0·77 ± 0·01	— —

* In μ/sec./volt/cm. units (see "Methods").
† The figures in parentheses give the number of replicate experiments.

down in the centrifuge, the supernatant fluid discarded and the interaction stopped by the addition of specific antiserum against the agent used. The amount of antiserum required for complete neutralization of the virus or RDE remaining on the cells was determined by previous titration. After standing for 15 min. at room temperature the cells were washed in two changes of normal saline. Agglutinability endpoints and electrophoretic mobility of the treated cells were determined as described under "Methods."

Table I gives the results for the three strains representing the main group of viruses. The agglutinability endpoints after the action of MEL, LEE or MIL do not significantly differ. Therefore the behaviour of the first group of viruses can be described by means calculated from the pooled results.

In the case of NDV and NDV_p the standard technique for stabilizing cells could not be applied, since it is known that a fraction of this virus does not elute spontaneously and is not removable by antibody (Burnet and Anderson, 1946). As far as the stopping of enzymic action is concerned omission of the treatment with antibody did not introduce any appreciable error because of the relatively long incubation period (16 hr.). As regards stabilization of cells, in most dilutions no special treatment was required as the cells had become stable by the end of the incubation period. Also, it was found possible to stabilize the cells which were still agglutinated by the addition of minute amounts of antibody.

The results presented in Table II show that the action of NDV and NDV_p is closely similar over the common range, the difference being only in the extent of

TABLE II.—*Agglutinability Endpoints during the Course of NDV and NDV_p Virus Action.*

Agent.	Agglutinability endpoint (weighted mean ± standard error) for							
	MU.	MU_p.	NDV.	NDV_p.	MEL.	PR8.	BEL.	LEE.
NDV (4)	0·80 ± 0·02	0·75 ± 0·02	0·76 ± 0·06	0·76 ± 0·06	0·73 ± 0·05	0·55 ± 0·06	0·46 ± 0·03	— —
NDV_p (2) . . .	0·82 ± 0·04	0·71 ± 0·05	0·87 ± 0·04	0·74 ± 0·05	0·78 ± 0·04	0·49 ± 0·04	0·41 ± 0·03	0·39 ± 0·02

complete action. The agglutinability endpoints fall in the same order as with the first group of viruses but the readings are consistently lower. A remarkable exception is the fact that agglutinability for NDV_p, which is lost between that of PR8 and BEL with all other agents, in this case disappears at the level of MEL and NDV before PR8, would be affected.

During the experiments with SW virus it was noticed that after treatment with small amounts of virus the cells behaved heterogeneously in the electric field, rendering the use of a simple average value questionable. The phenomenon was further investigated and will form the subject of a later paper. The possible error introduced by the heterogeneity could be controlled by using also the alternative method, viz., treatment of cells with larger amounts of virus for different periods of time.

The experimental findings (Table III) indicate that the order in which the agglutinability endpoints fall corresponds to that found with the group MEL-LEE-

MIL. Respective endpoints are intermediate between the first group and NDV throughout.

TABLE III.—*Agglutinability Endpoints during the Course of SW Virus Action.*

Agent	Agglutinability endpoint (weighted mean ± standard error) for									
	MU.	MU_p.	NDV.	NDV_p.	MEL.	PR8.	BEL.	LEE.	SW.	MIL.
SW (10) . .	0·83 ± 0·03	0·81 ± 0·02	0·86 ± 0·02	0·77 ± 0·02	0·84 ± 0·02	0·80 ± 0·02	0·64 ± 0·01	0·60 ± 0·02	0·53 ± 0·01	0·42 ± 0·02

With the " receptor destroying enzyme " (RDE) of *V. cholerae* again a standard time was used and the enzyme diluted. To make more closely spaced readings possible two sets of interdigitating doubling dilutions were employed.

TABLE IV.—*Agglutinability Endpoints during the Course of RDE Action.*

Agent.	Agglutinability endpoint (weighted mean ± standard error) for									
	MU.	MU_p.	NDV.	NDV_p.	MEL.	PR8.	BEL.	LEE.	SW.	MIL.
RDE (7) . .	0·44 ± 0·02	0·42 ± 0·02	0·81 ± 0·03	0·58 ± 0·01	0·74 ± 0·04	0·65 ± 0·03	0·39 ± 0·01	0·39 ± 0·02	0·39 ± 0·01	0·25 ± 0·02

As in previous experience (Burnet, McCrea and Stone, 1946 ; Stone, 1947), mumps virus, instead of being on top of the gradient, falls between NDV_p and BEL when cells are treated with RDE. The order of the rest of the viruses is identical with the gradients established with the other agents. The electrophoretic mobility values are consistently and significantly lower than the corresponding readings with the first group of viruses.

Mobility and Agglutinability Patterns after Additive Partial Treatments.

The evidence presented in the first part of this paper, suggesting that the two actions of the NDV-SW-RDE group proceeded simultaneously, warranted a closer study of the process. A suitable method was available in interrupting receptor destruction at any chosen level and analysing the effect with the aid of a second agent.

Both techniques of obtaining partial treatment, as used in the previous section, could be applied. The usual practice was to set out serial dilutions of the virus, and incubate these with a standard amount of red cells for a period of time determined in preliminary tests. Enzymic interaction was stopped by the addition of specific antibody as above. The washed cells were then divided into several lots and incubated with the second agents ; the amount of virus used for the second treatment was sufficient to produce maximal action on the control cells. Of the viruses with additional action NDV could not be used as first agent in these experiments because it cannot be completely removed from red cells by antibody.

Although there was good agreement between parallel tests it is impracticable to pool the results, as the exact levels at which the first actions were stopped varied slightly from one experiment to another. The Tables therefore give one representative experiment of each kind only, and contain both electrophoretic mobility readings and the corresponding patterns of agglutination with different

test-strains. Only those reactions are presented where there was a significant further change in electrophoretic mobility and agglutinability on second treatment. In the actual experiments the lower dilutions of the first agent reduced electrophoretic mobility to such an extent that the second agent was not adsorbed to these cells and did not cause any further alteration in mobility or agglutinability. These negative results are omitted from the Tables.

The partial actions of RDE followed by LEE (Table V) show that the final level of electrophoretic mobility is a function of the extent of RDE-action, clearly

TABLE V.—*Partial Treatments with RDE followed by Complete Action of LEE.*

Electrophoretic mobility* after		Final agglutination pattern† against			
First treatment.	Second treatment.	BEL.	LEE.	SW.	MIL.
0·50	0·47	−	−	−	++
0·70	0·51	−	−	±	++
0·98	0·63	−	tr.	±	++
1·11	0·72	−	±	++	++
1·20	0·74	−	+	++	++
Control‡	0·77	−	+	++	++

* In μ/sec./volt/cm. units.
† Against ≡ 5 agglutinating doses of the test viruses.
‡ No first treatment ; electrophoretic mobility = 1·30 μ/sec./volt/cm.

demonstrating the progressive " second type " activity of RDE. The remarkable feature of the agglutination results is the finding that LEE virus, which on its own does not remove agglutinability for itself, destroys receptors for both LEE and SW if preceded by a more extensive action of RDE.

The action of SW superimposed on partial actions of RDE (Table VI) differs notably from LEE action, both in that the final electrophoretic mobility levels are identical irrespective of the extent of the first treatment and that the trend

TABLE VI.—*Partial Treatments with RDE followed by Complete Action of SW.*

Electrophoretic mobility after		Final agglutination pattern against		
First treatment.	Second treatment.	LEE.	SW.	MIL.
0·41	0·35	−	−	++
0·52	0·35	−	−	++
0·58	0·35	−	−	±±
0·70	0·37	−	−	+
0·84	0·36	−	−	±
0·97	0.37	−	−	tr.
1·09	0·38	−	−	tr.
Control	0·39	−	−	±

TABLE VII.—*Partial Treatments with RDE followed by Complete Action of NDV_p.*

Electrophoretic mobility after		Final agglutination pattern against			
First treatment.	Second treatment.	BEL.	LEE.	SW.	MIL.
0·50	0·31	−	−	+	++
0·70	0·36	−	−	+	++
0·98	0·37	−	−	+	++
1·11	0·37	−	−	±±	++
1·20	0·36	−	−	±±	++
Control	0·35	−	−	±±	++

in the agglutinability pattern is the reverse. The characteristics of the agglutinability results are better evaluated after discussion of the underlying mechanisms and are therefore relegated to the end of the paper.

NDV_p following partial RDE treatments (Table VII) reaches electrophoretic mobility values indistinguishable from its own complete action. Similarly, the differences in agglutinability patterns are insignificant.

When SW was used as first agent, the added action of LEE (Table VIII) produced a picture resembling that of the RDE-LEE combination. The trend is

TABLE VIII.—*Partial Treatments with SW followed by Complete Action of LEE.*

Electrophoretic mobility after		Final agglutination pattern against			
First treatment.	Second treatment.	BEL.	LEE.	SW.	MIL.
0·64	0·65	−	−	±±	++
0·80	0·75	±	±	±±	++
0·95	0·76	−	+	±±	++
1·05	0·77	tr.	+	++	++
1·11	0·77	−	+	++	++
Control	0·78	−	+	++	++

similar both in mobility and agglutinability changes but the differences are on a smaller scale.

NDV_p following the partial action of SW (Table IX), on the other hand, showed definite gradation in the mobility endpoints, the final levels being the higher the

TABLE IX.—*Partial Treatments with SW followed by Complete Action of NDV_p.*

Electrophoretic mobility after		Final agglutination pattern against			
First treatment.	Second treatment.	BEL.	LEE.	SW.	MIL.
0·64	0·52	−	−	+	++
0·80	0·47	tr.	−	±±	++
0·95	0·41	±	±	±±	++
1·05	0·39	−	−	++	++
Control	0·37	−	−	±±	++

more extensive the pre-treatment with SW. The agglutination reactions nevertheless are practically identical throughout.

DISCUSSION.

The experimental evidence given above is in general agreement with our working hypothesis concerning the physical changes which result from viral action on erythrocytes. Most viruses of the mumps-influenza group reduce electrophoretic mobility *pari passu* with agglutinability, thus forming a series identical with the " receptor gradient " of Burnet, McCrea and Stone (1946). The two exceptions, swine influenza and Newcastle disease viruses, lower mobility to a degree exceeding that expected from their effect on agglutinability. The soluble enzyme prepared from *V. cholerae* filtrates, RDE, " destroyed receptors " for all virus strains, and at the same time reduced electrophoretic mobility to values below any of the viruses. We postulated therefore that the cellular surface contained substrate groupings of different types, substrate A being available for all viruses to an

extent determined by their position in the receptor gradient, substrate B for SW and NDV only; RDE was supposed to act on both, as well as on additional groups termed C.

The main question we sought to answer in the present study was whether these enzyme actions proceed simultaneously or consecutively. The course of each reaction was analysed therefore by interrupting the process at closely-spaced intervals and determining the corresponding levels of electrophoretic mobility and agglutinability. Further information could be derived by superimposing the action of one virus on the partial action of another. If the mobility of cells treated successively with two agents is significantly lower than that obtained with the second agent alone, it follows that the difference in mobility is due to the action of the first agent on substrates not available to the second. This method can be applied to any pair of viruses : since it makes use of partial treatments it does not demand that the second agent be below the first in the agglutination gradient, as was the case in our previous work on double treatment (Stone and Ada, 1950).

Enzymic action was interrupted at the required stage by adding specific antibody, known to remove virus from cells. The " cell sensitizing agent " of mumps and Newcastle disease viruses, which is not taken off by this means, imposed certain technical limitations on the use of these viruses.

A limitation of another kind is inherent in the method of determining agglutinability endpoints. Unlike the measurement of electrophoretic mobility, agglutinability is assessed on a discontinuous scale, and readings can be made only at the fixed points where one of the test-viruses gives a standard agglutination pattern. Furthermore, the fundamental relationship of the two changes in the behaviour of erythrocytes, viz., electrophoretic mobility and agglutinability, is unknown at present, and will probably remain so until they can be described in terms of substrate acted upon or end-product released. Thus while the results give satisfactory estimates of the additional action of SW, NDV and RDE at the individual agglutinability endpoints, no direct conclusion can be drawn from the experimental data as to the nature of the underlying chemical processes.

However, one type of change can be profitably evaluated against the other. The procedure is to select a method of plotting which will give a straight line for the agents acting only on the assumed section A. This is achieved simply by using the "agglutinability endpoints" (electrophoretic mobility value at which cells give partial agglutination with 5 agglutinating doses of the respective virus), determined in tests with these agents, to represent the individual viruses on the abscissa. In this way the curve for the A-section viruses is, by definition, a straight line with a slope of unity intercepting the ordinate at the mobility value of normal red cells (1·30 μ/sec./volt/cm.). Plots of the other agents (SW, NDV, RDE) might take any shape. If they are of the type $y = a + bx$, i.e., straight lines, it follows that the enzymic actions on both section A and B have the same kinetics ; if the equation describing any one curve is of a different order, it has to be concluded that the kinetics of the two reactions are dissimilar. If a curve intercepts the " A-substrate-line" at $y = 1{\cdot}30$, the action on substrates A and B starts simultaneously ; if it diverges from line A at a later point, it means that action on substrate B starts at a later time which can be characterized by the corresponding co-ordinates. The slope of the curves at any point will show directly the rate of action on substrates A and B, bearing in mind that the rate on section A is by definition unity ($b_A = 1$).

The Figure is constructed from the experimental data of both the present and two previous papers (Ada and Stone, 1950 ; Stone and Ada, 1950). It is evident that the average agglutinability endpoints of the different agents plotted against the corresponding electrophoretic mobilities give straight lines which differ from each other only in their slopes. Before drawing conclusions, however, it is

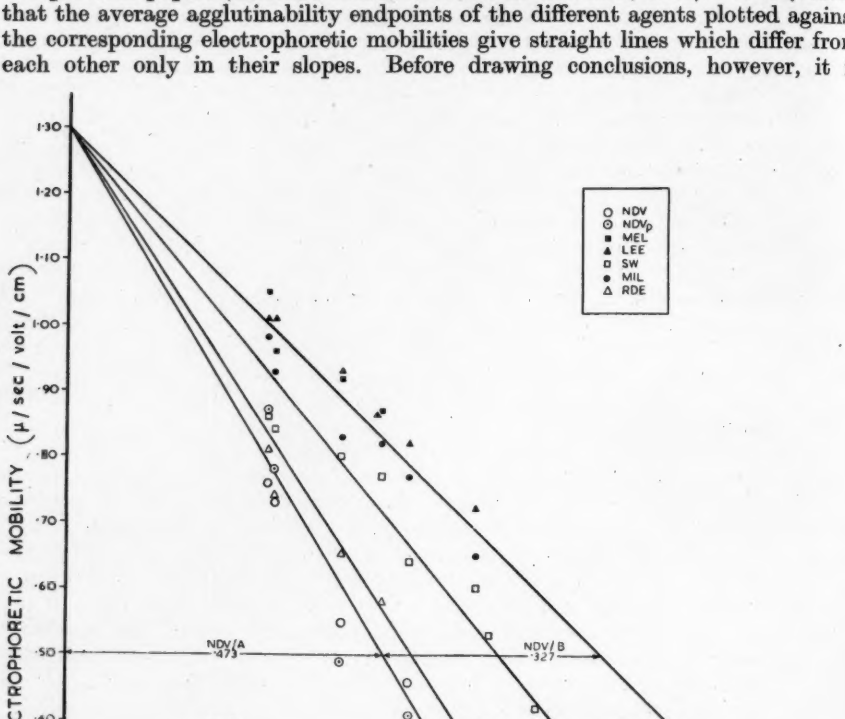

FIGURE.—Relationship between agglutinability and electrophoretic mobility.

advisable to examine the statistical validity of the results. There are four questions to be answered : (1) Are all the regression curves legitimately expressed as straight lines ? (2) Do they cut the Y-axis at a common point ? (3) Are the regression coefficients determined accurately enough to allow estimation of relative actions on the two types of substrates ? (4) Are the differences between the behaviour of the various agents significant ?

The test of deviation from linear regression* gave an unequivocal answer showing, in all instances, no more than random sampling departures ($\cdot 6 > P > \cdot 2$). Thus, when plotted on a scale ensuring a straight line for *one* group of agents, *all* regression curves were found to comply with the most stringent requirements of linearity.

Evaluation by Student's t-test of the constants a from equation $y = a + bx$ showed that none differed significantly from 1·30. Hence a set of straight lines intercepting the ordinate at the mobility value of normal red cells is truly representative of the experimental findings.

As all curves have the same origin, the regression equation may be written $y = bx$, and the regression coefficient $b = \Sigma xy / \Sigma x^2$. Calculating this value and its standard error we obtain $b_{SW} = 1{\cdot}238 \pm 0{\cdot}034$; $b_{RDE} = 1{\cdot}553 \pm 0{\cdot}044$; $b_{NDV} = 1{\cdot}690 \pm 0{\cdot}057$. Since all regression coefficients carry an error of less than $\pm$ 5 per cent, a fairly accurate estimate can be made of the action of any virus on sections A and B, using the formula $X_A = Y/b$ and $X_B = Y(b-1)/b$, where Y is the reduction of electrophoretic mobility when the respective agent has acted to completion. The values, as also shown in the Figure, are: SW ($X_A = 0{\cdot}750$, $X_B = 0{\cdot}179$, $SE_X\dagger = 0{\cdot}026$); RDE ($X_A = 0{\cdot}727$, $X_B = 0{\cdot}402$, $SE_X = 0{\cdot}021$); NDV ($X_A = 0{\cdot}473$, $X_B = 0{\cdot}327$, $SE_X = 0{\cdot}016$); NDV_p ($X_A = 0{\cdot}562$, $X_B = 0{\cdot}388$, $SE_X = 0{\cdot}019$).

An Analysis of Variance worked out for the regression coefficients (with the assumption that $a = 1{\cdot}30$, on the evidence presented above) showed that the values for RDE and NDV were not distinguishable ($P > \cdot 1$); for all other combinations of two agents the differences in the slope of the regression lines were highly significant ($P < \cdot 01$).

In the light of these findings the additional actions of SW, NDV and RDE can be interpreted in the framework of a simpler and more comprehensive hypothesis than earlier. There now seems to be no need to postulate a "section C," which was thought to be acted upon by RDE and probably NDV_p. Several facts are better explained by the existence of a gradient along section B, similar to that recognized for section A. For example, the additional actions of RDE and NDV_p are indistinguishable in extent, and the slope of their curves, instead of giving any hint of a third type of action, remains constant and identical with that of NDV. This gradient on section B can be defined in the same terms (viz., electrophoretic mobility), as has been done for section A. Thus, the total extent of section B is equivalent to a reduction of electrophoretic mobility of 0·40; of this SW can do 0·19, NDV 0·33, NDV_p 0·39 and RDE 0·40.

Both the direct observational and statistical evidence show that the actions on the two sections start simultaneously and proceed in parallel. This would suggest the duplicity of attackable substrate-types rather than two types of enzyme. Were there two enzymes acting on the same substrate no linear regression could be expected. The third possibility, two types of enzyme acting on two substrates, would fit the experimental facts only by making the highly unlikely assumption of identical Michaelis-constants. Accepting the first interpretation, the regression coefficients attain a real meaning inasmuch as they are

* Standard statistical methods, as set out by Fisher (1948), were used for all computations.

† The standard error of X_A and X_B equals $SE_b . Y/b^2$. It is recognized that there is a theoretical difficulty in inverting the regression equation, but for practical purposes the expression given for SE_X is still the best available.

the measure of relative affinity determining the distribution of an enzyme between two substrates. By the same token it is more appropriate to say that the viruses in the " A-substrate-group " have such low affinity for substrate B that under the experimental conditions there was no demonstrable action.

The part the two substrates play in holding the virus particle at the cell surface can be evaluated by a closer analysis of the additive actions. If only substrate A were acting as adsorptive force, agents with a second action should reduce electrophoretic mobility to a lesser extent when following partial action of a member of the A-substrate-group. If the virus were held by substrate B also then the final electrophoretic mobility value should be constant, but the agglutinability patterns should vary with the nature of the agent employed for the first action. Such differences were looked for and consistently found. Examples of the class held by substrate A but not by substrate B are NDV, NDV_p, MEL (cf. Tables VII and IX), and probably BEL and PR8. The second class is typified by SW. It is worth noting, however, that both MIL and LEE cause greater loss of agglutinability when preceded by an agent acting on section B, and that SW and RDE are capable of completely removing the agglutinability for LEE and MIL which these viruses themselves cannot do. These facts would suggest that substrate B contributes to the force holding LEE and MIL, though these viruses lack specific action directed against that substrate.

While providing a coherent picture of the activities on erythrocytes of influenza and Newcastle disease viruses and the " receptor destroying enzyme " of *V. cholerae*, this study did not enable us to fit mumps virus into the general pattern. Technical limitations are only partly responsible for this failure, since such well-established facts as the strikingly different position of mumps virus in the gradient when determined by RDE or by one of the influenza viruses still remain unexplained.

SUMMARY.

The relationship of electrophoretic mobility and agglutinability of erythrocytes treated with viruses of the influenza group or with the " receptor destroying enzyme " (RDE) of *V. cholerae* was investigated by two methods.

The majority of these viruses falls in a single group in showing only one type of enzymic action.

The viruses of Newcastle disease and swine influenza as well as RDE have an additional action which probably belongs to the same enzyme but is directed against a different substrate.

All agents act to characteristically different levels on the first substrate, and a similar gradient exists also for the second, the latter being $SW < NDV < NDV_p < RDE$. Evidence is presented that those agents which act on both substrates do so simultaneously.

The theoretical implications of the results are discussed and the earlier hypothesis simplified and extended.

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URAEMIA IN THE RABBIT AFTER INJECTION OF CRYSTALLINE MYOGLOBIN.

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THE observation that injuries to muscular masses are followed by an excretion of urine containing a red-brown pigment has been reported by many authors (Yuile and Clark, 1941 ; Bywaters and Beall, 1941) but the mechanism of this phenomenon remained obscure until Bywaters and his colleagues (Bywaters and Beall, 1941 ; Bywaters and Delory, 1941 ; Bywaters, Delory. Rimington and Smiles, 1941 ; Bywaters, 1942, 1943 ; Bywaters and Stead, 1944) reported their findings in man. They identified the pigment as myoglobin and also demonstrated the rôle played in its effect on the kidneys by the urinary pH, a phenomenon already known as far as the excretion of human haemoglobin was concerned (Baker and Dodds, 1925 ; Bing, 1944). Bywaters and Popjak (1942) studied the mechanism of the action of myoglobin in rabbits. They produced muscular damage by prolonged compression of the limbs, but the effects of these injuries were not exactly comparable with those observed in man. In particular the elimination of myoglobin in urine was never observed. In a later report Bywaters and Stead (1944) concluded that rabbit muscles had a low myoglobin content, and that a myoglobin concentration above the renal threshold could not therefore be obtained. To prove this hypothesis they injected solutions of human myoglobin prepared according to a modified Theorell (1932) method, and obtained results which were analogous to those observed in man. The experiments of Bywaters and his colleagues can be summed up briefly as follows : (*a*) An injection of human myoglobin into a normal rabbit with alkaline urine produces an increased diuresis immediately following the injection, with raised concentration of urea and creatinine in the urine and a transitory rise of blood urea concentration. (*b*) In animals fed with acidifying diet, with urine pH ranging from 5 to 6, a similar injection of myoglobin produces (1) an increased diuresis with elimination of a brown precipitate about 7 hours after the injection, (2) a progressive rise in blood urea which in some cases reaches values as high as 300 mg. per 100 ml., (3) an increase of blood creatinine, (4) a subsequent fall in diuresis progressing to a total renal block.

Since they did not have pure crystalline myoglobin, these workers used a mixture of myoglobin and muscle protein in equal parts as inferred from relative contents of N and Fe, and they took care to point out that the administration of an impure substance might alter or complicate the process owing to the presence of heterogeneous and unknown components. We have therefore carried out experiments on the action of pure crystalline myoglobin in producing a renal block under the same experimental conditions.

MATERIAL AND METHODS.

Myoglobin was obtained from horse muscle following the method devised for preparation of crystalline myoglobin from human muscle (Rossi-Fanelli, 1949). The crystals were washed several times with a saturated ammonium sulphate solution. Further purification was obtained by repeated sedimentation of crystals in clean $(NH_4)_2SO_4$ saturated solution and decantation of supernatant until a constant iron content was obtained. Iron was determined by the α-α dipyridil colorimetric method (Hill, 1930), and was found in two samples of myoglobin used to be 0·341 and 0·343 per cent respectively. The crystalline myoglobin was kept in a saturated $(NH_4)_2SO_4$ solution in the ice box for a period not longer than two months, when partial denaturation occurred.

The crystals were separated by centrifugation, dissolved in a minimum quantity of distilled water and dialyzed against Ringer solution until complete disappearance of SO_4 ions. Excess fluid was then removed by ultrafiltration. All these manipulations were carried out at 0°, and the isotonic solution thus obtained was sterilised by filtration through a Berkfeld candle. The myoglobin content of this solution, which could be kept sterile for some days at 0°, was determined (total N $\times$ 6·25), controlling the data by comparison with iron content.

The experiments were carried out on 10 rabbits, average weight 2·1 kg., of which 7 were treated and 3 served as controls. The controls were fed with normal mixed diet; the other animals were given an acidifying diet composed mainly of oats, and in addition 120 ml. of 0·0017 N HCl were administered daily to each of them. This diet lowered the urinary output, as compared with the controls, which was probably due to a lower water content of the diet. No other differences were noted. The pH of the urine fell gradually, reaching values between 5 and 6 in 5–7 days.

Urea, nitrogen and creatinine were determined in the blood and urine of each animal. The ammonia content of the urine was calculated indirectly by difference after absorption with permutite. Urea was determined by the Van Slyke-Cullen urease method with aeration and titration, and creatinine by the Folin method using sodium picrate.

Myoglobin was injected intravenously at various concentrations, the total volume being always 10 ml. Blood for determinations was collected from the ear vein of the opposite side immediately after the injection and then at fixed intervals.

The examination of the urine samples included an examination of sediment. When the injection was followed by albuminuria or myoglobinuria the determination was carried out on urine ultrafiltrate. The presence of myoglobin in the urine was ascertained both by the benzidine tests and by spectrophotometry.

After the death of each animal the kidneys were removed, weighed and fixed for histological examination. The urine found in the bladder, if any, was collected and examined as usual.

RESULTS.

The results observed in the three control rabbits are in close agreement with each other and with the results reported by Bywaters and his colleagues. They can be summed up as follows: An injection caused a state of excitement, sometimes accompanied by shivering. After disappearance of these symptoms the animals remained prostrate for about half an hour and then recovered.

Considerable diuresis set in 12–15 min. after the injection, and the urine contained myoglobin, which could be demonstrated spectrophotometrically. The urine was alkaline, the pH being about 7·8.

Two of the animals showed albuminuria. The total volume of urine passed during the day was above normal and its concentration was only slightly reduced; thus the quantity of urea N, total N and creatinine excreted was increased. The data are reported in Tables I and II.

TABLE I.—*Injection of Myoglobin into Rabbits kept on Normal and Acidifying Diets, Dosage of Myoglobin, pH of Urine, Effect on Weight of Kidneys.*

	Controls.			Animals on acidifying diet.						
Animal No.	1.	2.	3.	4.	5.	6.	7.	8.	9.	10.
Body weight (kg.)	2·30	2·25	2·03	2·25	2·05	2·12	2·18	2·22	2·17	2·32
Myoglobin injected (mg.)	110·00	124·00	110·00	97·50	100·50	110·50	114·00	105·00	103·00	121·00
Myoglobin injected per kg. body weight	48·00	55·00	54·00	43·00	50·00	52·00	52·00	45·00	47·00	52·00
pH of urine	7·80	7·80	7·80	6·00	5·90	5·90	5·90	5·90	6·00	5·87
Weight of right kidney (g.)	6·80	7·50	7·20	11·20	12·10	11·60	11·30	11·20	11·90	11·70
Weight of left kidney (g.)	7·00	7·60	7·14	11·40	12·40	11·90	11·02	11·50	12·01	12·10
Average kidney weight	7·37			11·63						

TABLE II.—*Injection of Myoglobin into Rabbits on Normal Diet, Changes in Creatinine, Total N and Urea N in Urine and Blood.*

		Urine levels (mg. per 100 ml.). Hours after injection of myoglobin.					Blood levels (mg. per 100 ml.). Hours after injection of myoglobin.				
	Animal No.	1.	2.	4.	7.	20.	1.	2.	4.	7.	20.
Creatinine	1	68	51	39	38	54	1·12	1·11	1·13	1·12	1·11
	2	69	40	30·6	38	54	1·21	1·22	1·21	1·18	1·18
	3	58	47	32	32	62	1·17	1·17	1·16	1·20	1·23
Total N	1	365	220	145	516	524	—	—	—	—	—
	2	362	382	220	395	495	—	—	—	—	—
	3	321	340	192	338	523	—	—	—	—	—
Urea N	1	324	179	106	463	507	12·4	14·1	14·0	14·2	11·8
	2	320	341	188	336	428	12·3	14·2	14·1	14·2	12·4
	3	294	298	157	279	473	12·7	13·3	14·6	14·4	12·5

No remarkable changes were observed in the blood of these three animals. Urea N increased by the end of the first hour after injection and remained stationary for one day. The creatinine content did not vary. After about 20 hr. all values returned to normal. The animals were killed 48 hr. after the injection and their kidneys were then examined (Table I).

In the seven animals on an acidifying diet the following results were obtained when the pH of urine reached values less than 6 and myoglobin was injected. The data are reported in Tables III and IV. No significant differences between individual animals were observed; on the contrary, response to the injection seemed to be particularly uniform and the course of the experiments can be represented schematically.

TABLE III.—*Blood Urea Changes following Injection of Myoglobin into Rabbits kept on an Acidifying Diet.* (*Blood urea N mg. per* 100 *ml.*)

Hrs. after injection myoglobin.	Animal No.						
	4.	5.	6.	7.	8.	9.	10.
0	7·95	12·00	10·07	11·60	11·24	14·31	12·21
2	10·40	12·70	14·00	12·80	18·22	20·18	14·96
4	20·40	15·70	16·60	22·50	31·56	26·40	35·91
6	22·50	18·30	20·50	68·00	41·00	30·00	—
8	20·90	23·00	24·70	78·90	84·00	50·42	49·60
10	40·31	—	—	—	—	—	—
12	—	30·05	26·30	80·00	85·03	59·12	64·12
14	70·17	—	—	—	—	—	—
16	95·93	40·31	29·00	78·50	90·40	67·50	—
18	—	—	—	—	—	—	69·74
20	105·12	45·00	30·00	85·40	90·70	79·14	—
22	—	—	—	—	—	—	—
24	105·70	61·45	35·90	93·40	94·00	98·71	84·12
26	129·48	70·07	49·07	105·20	113·00	103·21	—
28	—	—	—	—	—	—	—
30	—	81·30	61·27	121·00	125·00	113·00	134·00
32	125·15	—	—	—	—	—	—
34	140·06	88·70	119·00	—	—	127·63	—
36	—	135·00	—	—	—	—	—
38	—	143·00	145·00	—	—	—	—

TABLE IV.—*Blood Creatinine Changes following the Injection of Myoglobin into Rabbits kept on an Acidifying Diet.* (*Blood Creatinine mg. per* 100 *ml.*)

Hrs. after injection.	Animal No.						
	4.	5.	6.	7.	8.	9.	10.
0	1·56	1·22	1·55	1·51	1·30	1·32	1·40
2	1·56	1·12	1·55	1·51	1·30	1·32	1·40
4	5·70	1·14	1·55	1·51	1·90	2·00	1·64
8	7·07	1·14	1·55	1·51	—	2·49	1·75
12	7·07	1·17	2·20	5·50	—	3·51	1·39
16	7·50	2·50	3·50	—	2·75	3·99	—
20	8·25	—	4·50	—	—	4·10	3·00
24	—	4·80	5·50	5·42	4·00	4·51	3·99
28	9·07	—	5·75	6·22	5·60	5·47	4·97
32	—	6·55	6·49	6·98	—	6·00	5·80
34	—	7·30	7·00	—	—	7·50	6·04
36	—	—	—	—	—	—	—
38	—	8·70	7·99	—	—	7·80	—

Seven rabbits of an average weight of 2 kg. were injected with 90–120 mg. crystalline myoglobin. There was usually no particular reaction after the injection, but the state of excitement persisted for a longer period in these animals than in the controls. A few minutes after the injection a conspicuous quantity of urine, usually clear and acid, was excreted.

In most cases this was followed by the elimination of scanty turbid urine stained reddish-brown, from which a reddish pigment precipitated. This urine contained myoglobin, as demonstrated by spectrophotometric examination.

In six rabbits a complete renal block set in after 2–3 hr. Rabbit No. 4 still passed some urine during the night; it was clear, as before the injection, but its pH was shifted towards the neutral point (pH 6·92).

The following alterations of the blood were noted after the injection: the concentration of urea N increased gradually, reaching values up to ten times

normal ; after 6–8 hr. the concentration of creatinine also began to rise ; maximum values were reached just before the death of the animal. All the animals died spontaneously from renal block 30–38 hr. after the injection.

DISCUSSION.

Summing up we may say that the results of Bywaters and his colleagues were fully confirmed, but in addition we demonstrated that pure crystalline myoglobin was responsible for the renal block in rabbits only if the urine pH was lower than 6.

Renal block, increase of blood urea and creatinine and death of the animal were observed in all cases, and were produced with quantities of crystalline myoglobin smaller than those used by Bywaters and his colleagues (90–120 mg.).

An interesting fact is the statistically significant increased weight of the kidney in animals fed with acidifying diet as compared with the controls. On microscopical examination of the kidneys only a very intense oedema was observed, and in particular the accumulation of pigment in the glomeruli and tubules was neither constant nor characteristic. Thus the mechanism of the renal block does not seem clear to us, and the data available do not allow us to accept the hypothesis of a block due to pigment precipitation in the tubules, as was suggested by Baker and Dodds (1925). It seems more probable to us that the renal insufficiency might be due to oedema and consequent ischaemia produced by an unknown mechanism.

SUMMARY.

When crystalline myoglobin was injected into normal rabbits whose urine was either neutral or slightly alkaline they showed some transitory changes in their urinary nitrogen excretion, but were fully normal 20 hours after an injection of crystalline myoglobin. Renal block was only produced when crystalline myoglobin was injected into rabbits which had been kept on an acidifying diet and whose urine was of a pH lower than 6. The microscopic examination of the kidneys showed intense oedema, but there was no constant finding of accumulation of pigment in the glomeruli and tubules.

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IN VIVO OBSERVATIONS OF THE EFFECTS OF CORTISONE UPON THE BLOOD VESSELS IN RABBIT EAR CHAMBERS.

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THERE appears to be general agreement among the few experimental workers who have studied the problem that cortisone exerts an inhibitory influence on the formation of new vessels during the process of inflammation and repair. Thus cortisone has been found to depress this activity in wounds of the skin of mice (Spain, Molomut and Haber, 1950), and of rabbits (Baxter, Schiller, Whiteside and Straith, 1951), in the healing of fractured femora (Blunt, Plotz, Lattes, Howes, Meyer and Ragan, 1950) and in skin-autografts in rabbits (Billingham and Krohn, 1951).

In the rabbit's eye cortisone has been found to inhibit the new vessel formation which follows the experimental injection of sodium hydroxide into the cornea (Jones and Meyer, 1950), and that which follows corneal burns (Lister and Greaves, 1951) and the intracameral injection of alloxan (Ashton, Cook and Langham, 1951).

That a similar inhibition may occur in man was indicated by the work of Creditor, Bevans, Mundy and Ragan (1950), who found almost no proliferating blood-vessels in skin biopsies taken from patients treated with adrenocorticotrophic hormone, and it has since been found by many other clinical investigators that cortisone has a powerful therapeutic action in the control of corneal vascularisation.

The mechanism whereby cortisone exerts this suppressive influence has so far remained unexplained, and this is not surprising since the factors which normally stimulate vascular growth are themselves imperfectly understood. In an endeavour to elucidate this problem we have carried out a microscopical study of the effect of cortisone upon the ingrowth of newly formed vessels into rabbit ear chambers. Although this was the sole purpose of our original project, the effects which were noted have a bearing upon other aspects of inflammation and repair, which cortisone is known to modify.

In this paper an attempt is made to assess the respective rôles played by the observed phenomena in regard to both new vessel formation and other components of inflammatory and reparative reactions.

EXPERIMENTS.

Perspex chambers were inserted into six rabbit ears using a technique similar to that described by Sandison (1928*b*). Twelve days after the insertion of the chamber, by which time a well-defined vascular network had been formed at the

periphery of the chamber, each rabbit received intramuscularly 20 mg. cortisone acetate daily in two divided doses. Daily observations were made on the ingrowing vessels, repeated serial photo-micrographs were taken of the progressive stages of vascular growth, and the mode and rate of the new vessel formation were compared with those obtained in control chambers. Since the study of new vessel growth does not lend itself to accurate measurement, the effects we have attributed to cortisone are those which our experience leads us to believe are beyond the range of normal variations. Indeed, it is upon such impressions, either clinical or experimental, that the previous contributions on this subject have been based.

EXPERIMENTAL FINDINGS.

The normal vascularisation of the rabbit ear chamber has been extensively studied by Sandison (1928*a*), and Clark, Hitschler, Kirby-Smith, Rex and Smith (1931), and our observations in the control animals were exactly as they have already described. The progression of the newly formed vessels across the chamber was marked by a zone of haemorrhage in advance of the distended capillary tips (Fig. 1); it was apparently formed by the extrusion of blood cells through the distended and fragile walls of the blind capillary sprouts, as a result of the constant pulsation transmitted from the general circulation through the feeding vessels. This phenomenon was constantly observed by Sandison (1928*a*), who considered it a regular accompaniment of new vessel formation. The dilated new capillaries soon became smaller in calibre, and formed pre- and post-capillary vessels and eventually arterioles.

In the animals receiving cortisone, however, a different picture gradually became apparent. A generalised vascular constriction involving both arterioles and venules occurred (Fig. 2 and 3), resulting in a reduced and often intermittent blood flow to the area of vascular new growth; the capillary network behind the advancing endothelial sprouts was, in consequence, comparatively empty. This effect became more evident as the period of cortisone administration extended, and was accompanied by a moderate delay in vascularisation of the ear chamber as compared with control animals. Endothelial budding occurred as in the normal animal, but a very large proportion of the capillaries so formed contained only plasma, in which a few almost motionless red cells were sometimes suspended (Fig. 4). The forcible ebb and flow seen in the newly formed capillaries of the control animal were conspicuously absent, and the zone of haemorrhage in advance of the capillary buds, which is so characteristic of normal new vessel formation, did not develop in the cortisone-treated animal.

Many of the larger vessels were markedly attenuated and showed an increased translucency of the vessel wall, suggesting an increased vascular tone (Fig. 5 and 6). Indeed, the vascular bed as a whole showed an exaggerated tonic response to extraneous stimuli and, following a slight stimulus (tapping the rabbit box), there was a complete and almost instantaneous shutting down of practically the whole of the vascular network. This effect was more marked than we have seen in normal animals.

While there was a moderate delay in vascularisation of the chambers in the test animals, this was to some extent more apparent than real, since the majority of the capillary buds and the vessels from which they arose were exsanguinated in the test chamber.

DISCUSSION.

The influence of cortisone upon new vessel formation.

A primary suppression of endothelial proliferation has been advanced as a possible causal factor in the inhibition of vascular new growth by cortisone (Duke-Elder and Ashton, 1951), but although such an action cannot be excluded in our experiments, the explanation of the differences between the control and test animals is rather to be sought in the modified circulatory conditions resulting from cortisone administration.

Thus the power of cortisone to reduce an abnormally raised capillary permeability, a property demonstrated by Cook and MacDonald (1951), could be responsible, in conjunction with the reduced circulation, for the absence of the haemorrhagic zone preceding the capillary tufts ; it would also play an important rôle in impeding the transfer of substances through the vascular wall, both to and from the surrounding tissues. It is known that immature vessels have an increased permeability (Abell, 1946), which promotes the passage of plasma proteins, hormones and enzymes into the surrounding tissue, and so provides a suitable medium for the advancing vascular growth and assists in the process of capillary penetration (Hadfield, 1951). By reducing this permeability, cortisone would tend to inhibit the progress of vascularisation.

Furthermore, the exsanguinated state of the growing capillary buds, resulting from the vascular constriction induced by cortisone, would tend to deprive the endothelial cells of oxygen and nutritional supplies essential to active mitotic division.

It is possible that the pulsation, to which the distended capillary tips are normally subjected, plays some part in the progression of new vessel formation, so that reduced blood flow, resulting from the vasoconstriction, may be a further factor in the inhibition of new vessel growth. It is also of importance to note that the degree of vasoconstriction slowly increases as the period of cortisone administration extends, and so leads, in accordance with Thoma's principle, to a gradual atrophy of a proportion of the capillary bed.

In summary, we conclude that cortisone is able to exert an inhibitory effect upon the rate and extent of vascularisation in the rabbit ear chamber, and this influence is more probably due to its profound effect upon the circulation than to a direct inhibition of endothelial growth. The increased vasomotor tone reduces both the volume and pressure of the blood supply, which gradually becomes inadequate either to promote or sustain the growth of new vessels, while the accompanying reduction in capillary permeability impedes the diffusion of substances favouring vascular growth and penetration.

The influence of cortisone upon inflammatory and repair processes.

The vascular constriction with its resultant reduction of blood circulation, shown in these experiments to follow the administration of cortisone, together with the known effect of cortisone upon capillary permeability, offer an attractive explanation for many of the clinical and experimental findings associated with the administration of cortisone in the fundamental reactions of inflammation and repair.

Capillary dilatation with accompanying stasis and increased capillary permeability are precursors of all inflammatory processes, and they constitute an

essential feature of the exudative phase. The increased capillary filtration is referable to vascular dilatation, in response to an arteriolar axon reflex and release of histamine-like substances, and to an increased capillary permeability resulting from the release of leucotaxine (Menkin, 1938), or other polypeptides (Spector, 1951).

The effect of cortisone upon capillary permeability, together with the vasoconstrictive action we have demonstrated, would prevent these vascular responses, and so inhibit both the exudative manifestations and leucocytic emigration in inflammation, and interfere with the demands of fibroblastic and other activities for increased nutritional supplies in repair.

During the course of our experiments Ebert and Wissler (1951) reported their observations on the effects of cortisone on the vascular reaction to large doses of horse serum using the rabbit ear chamber technique. As in our experiments, cortisone appeared to produce a marked increase in arteriolar tone, both in rabbits receiving horse serum and in normal controls. During the period of cortisone administration the characteristic allergic vascular reaction was reduced, if not completely abolished. That the same phenomenon occurs in man may be seen in the vasoconstrictive action of cortisone upon superficial corneal vascularisation (Fig. 7 and 8), and in its blanching effect upon the engorged synovia in rheumatoid arthritic joints (Hollander, Stoner, Brown and DeMoor, 1950).

Restricted space does not permit a review of all the relevant literature, nor a detailed discussion of the evidence upon which our conclusions are based—these will be presented in a paper to be published elsewhere—meanwhile we advance the hypothesis that the modifying action of cortisone in inflammation and repair,

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DESCRIPTION OF PLATES.

FIG. 1.—Vascularising transparent chamber. Control rabbit. The progression of the newly formed vessels across the chamber was marked by a zone of haemorrhage in advance of the distended capillary tips. The haemorrhage did not develop in the animal treated with cortisone. × 504.

FIG. 2.—Vascularising transparent chamber. Cortisone rabbit. First day of treatment. The cortisone effect is not yet apparent and the vessels are distended and engorged with blood as in the normal chamber. Compare with Fig. 3. × 88.

FIG. 3.—Same field as shown in Fig. 2. Twelfth day of treatment. The vessels now show marked constriction, particularly of the arterioles and capillaries. Note the increased translucency of the vessel walls. Compare with Fig. 2. × 88.

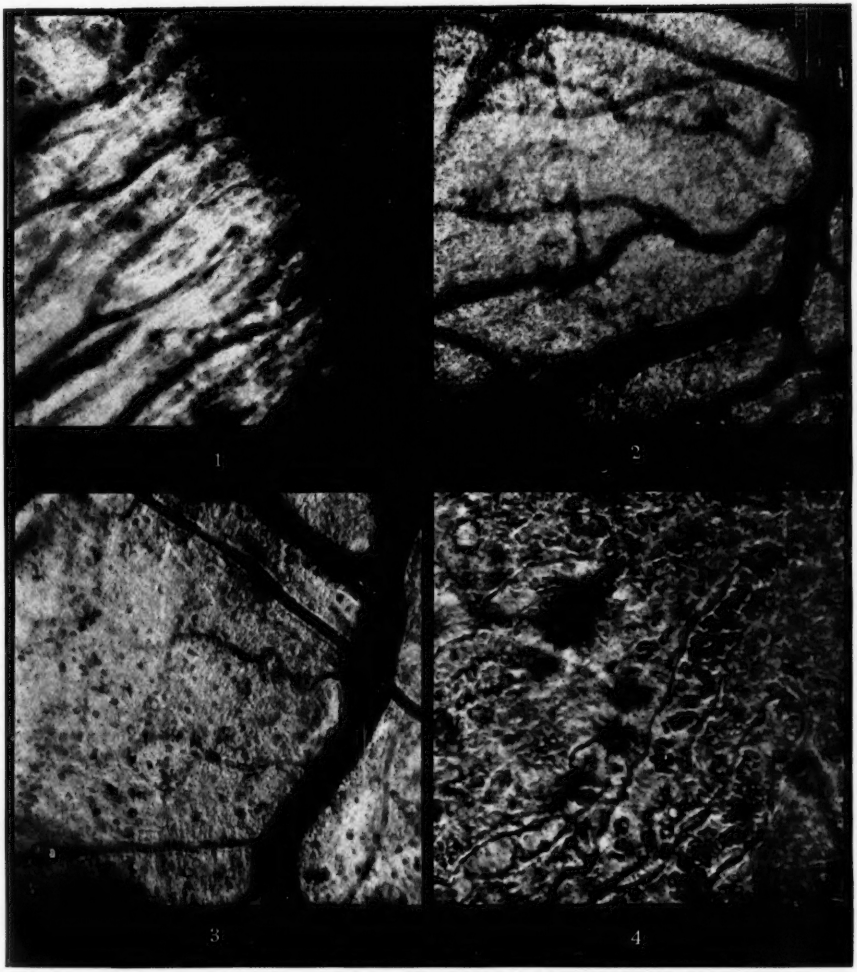
FIG. 4.—Vascularising transparent chamber. Cortisone rabbit. Fifteenth day of treatment. The majority of the capillary buds contained only plasma in which a few almost motionless red cells were sometimes suspended. × 504.

FIG. 5.—Vascularising transparent chamber. Cortisone rabbit. Eighteenth day of treatment. There is generalised vasoconstriction with increased translucency of the vessel walls and the newly formed capillaries are partially or totally exsanguinated. × 212.

FIG. 6.—Vascularising transparent chamber. Cortisone rabbit. Twenty-second day of treatment. Endothelial budding occurred as in the control animal, but note the marked vasoconstriction leading to exsanguination of the capillary buds. The zone of haemorrhage shown in Fig. 1 is absent in the animal treated with cortisone. × 120.

FIG. 7.—Superficial corneal vascularisation in a patient with bilateral superficial keratitis. Before treatment with cortisone. Compare with Fig. 8.

FIG. 8.—Same eye as shown in Fig. 7, 24 days after treatment with subconjunctival injections of cortisone. Note the extensive shutting down of the capillary bed with marked vasoconstriction of the persisting vessels. The appearances are exactly as seen in the rabbit ear chamber experiments.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particularly in the former, stems in the main from its ability to promote vascular tone and to maintain normal vascular permeability.

The site and mode of action of cortisone in the production of these vascular changes are as yet unknown, and since the mechanisms involved are extremely complex, there are many ways in which cortisone might achieve its effects. However, the fact that cortisone, whether administered topically or systemically, is able to act with equal facility indicates the operation of a local mechanism.

As regards its effect on vascular tone, it may be that cortisone potentiates the action of vaso-constrictor agents such as noradrenaline, either by enhancing the sensitivity of cells to them, or possibly by blocking, inactivating or destroying amine oxidase. Alternatively it may operate by influencing circulating vasopressor substances, such as vaso-excitor material (Shorr, Zweifach and Furchgott, 1945, 1947 and 1948).

Such explanations are, of course, highly speculative, but they serve to indicate a few of the numerous mechanisms which may be involved, and to emphasize our conclusion that future research work, attempting to elucidate the site of action of cortisone in inflammation and repair, is most likely to be rewarded if directed towards a study of its influence upon vasomotor mechanisms.

SUMMARY.

The effect of cortisone upon the ingrowth of newly formed vessels has been observed in transparent chambers in the rabbit ear.

The most striking features noted in the vascularising test chambers were generalised vascular constriction, increased translucency of the vessel walls and an exaggerated tonic response to extraneous stimuli. Endothelial budding occurred as in the normal animal. While in the animals treated with cortisone there was a moderate delay in vascularisation, this was, to some extent, more apparent than real, since the majority of the capillary buds and the vessels from which they arose were exsanguinated.

It is concluded that cortisone is able to reduce the rate and extent of vascularisation in the rabbit ear chamber, and this influence is more probably due to its profound effect upon the circulation than to a direct inhibition of endothelial growth.

The generalised vascular constriction together with the depression of capillary permeability which result from cortisone administration are thought to be the factors of greatest importance in the suppression of the inflammatory reaction, and to have a significant bearing upon the inhibition of repair processes.

The site and mode of the action of cortisone in the production of these vascular changes are as yet unknown. Reasons are given for suggesting that future inquiry might profitably be directed towards a study of the influence of cortisone upon vasomotor mechanisms.

We are grateful to Dr. A. G. Sanders for invaluable instruction in the technique of inserting transparent chambers into rabbits' ears, and to Sir Stewart Duke-Elder, Dr. N. Ambache and Dr. B. Lacey for helpful criticism. Our thanks are due to Dr. Peter Hansell for the photomicrographs, to Miss E. FitzGerald for secretarial assistance, and we are indebted to Mr. N. Roberts for making the perspex chambers.

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THE EFFECTS OF LIPID EXTRACTION ON THE PROPERTIES OF IMMUNE SYSTEMS.

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ANTIBODIES which combine specifically with antigen but fail to give the characteristic visible reactions, i.e., precipitation or agglutination, are commonly referred to as " incomplete." The presence of such incomplete antibodies in immune sera has often been surmised, and has been actually demonstrated in the case of horse antisera to egg albumin (Pappenheimer, 1940 ; Heidelberger, Treffers and Mayer, 1940), rabbit serum globulin (Treffers, Heidelberger and Freund, 1947) and human serum albumin (Gitlin, Davidson and Wetherlow, 1949). Incomplete antibodies were also found to constitute a fraction of a precipitating rabbit serum to egg albumin (Heidelberger and Kendall, 1935). In human beings the incomplete Rh antibodies and the " reagins " responsible for allergic reactions have received much attention.

The failure of these antibodies to form precipitates has been ascribed to " univalence "; that is, their ability to combine only with *one* instead of the usual two molecules of antigen required for lattice formation. It has been claimed that various forms of treatment of antisera (heat, formaldehyde, ketene, etc.) transform precipitating antibodies into incomplete ones. Of these treatments the extraction of lipids in the cold with alcohol and ether is of particular interest, since there is no obvious reason why this method should reduce the valency of the antibodies.

The loss of flocculation following lipid extraction was first noted by Hartley (1925) working with rabbit antiserum to horse serum. To prevent precipitation completely he found it necessary to extract the antigen as well as the antibody. Precipitation could not be restored by the addition either of the ether soluble components or of normal rabbit serum. Horsfall and Goodner (1935, 1936) extracted the lipids from horse and rabbit antipneumococcal serum, thereby destroying their ability to agglutinate or precipitate. Although the addition of the extracted material had no effect, minute amounts of cephalin or lecithin added to the rabbit and horse antisera, respectively, completely restored their agglutinating properties. Tayeau, Faure, Neuzil and Pautrizel (1949) report that extracted non-flocculating rabbit antisera regain their ability to precipitate upon treatment with ninhydrin. Krueger and Heidelberger (1950) found that extracted horse antisera to rabbit serum albumin and globulin and Type III pneumococcus flocculate more slowly than the untreated sera, but that with longer periods of standing and higher speeds of centrifugation than usual complete precipitation is obtained. The discrepancy between these results and those of previous investigators is attributed to their failure to use quantitative analytical methods, and to observe the precautions of longer standing and more effective centrifugation.

This study was undertaken to find out whether incomplete antibodies can be prepared by extracting the lipids from precipitating antisera, and to compare the behaviour of these artificial incomplete antibodies with the naturally occurring ones. Quantitative studies of their ability to inhibit the precipitation of homologous untreated antibody and antigen or to co-precipitate with such a system should give an indication of any valency changes that have taken place as a result of delipidation.

MATERIAL AND METHODS.

Sera used were rabbit antisera to crystalline egg albumin, crystalline horse serum albumin, and horse globulin ; and horse antiserum to Type I pneumococcus. Quantitative precipitin curves were determined by adding increasing amounts of antigen to constant volumes of antiserum. All tubes in a given series were brought to constant vol. with saline and left at room temp. until they flocculated or for at least 1 hr. After standing at + 4° for at least 1 day, the ppts. were centrifuged at 3000 r.p.m. and washed 3 times with 3 ml. chilled saline. Protein was estimated by the Folin-Ciocalteau method as modified by Heidelberger and MacPherson (1943).

Extraction of lipids was done essentially according to Horsfall and Goodner's (1935) methods A and B. The chilled antiserum was added slowly and with stirring to 10 vol. absolute ethanol at − 10°, and shaken for 6 hr. at − 10°. After centrifugation in the cold the process was repeated with the same vol. of fresh alcohol, and shaking continued for 10 hr. at − 10°. Rabbit antisera were then extracted with petroleum ether (10 hr. at − 10), followed by anhydrous ether once at − 10° for 10 hr. and twice at room temp. for a total of 12 hr. (Method B). Horse antisera were extracted similarly (Method B) or without the petroleum ether treatment (Method A). After the last centrifugation ether was removed in a vacuum desiccator. All sera were dissolved in their original vol. of saline, and proved perfectly soluble.

Alcohol and ether extracts were pooled, concentrated to dryness *in vacuo* and re-constituted to the original serum vol. with saline ; the residue was only partially soluble and a cloudy suspension was obtained.

The globulin fractions of the extracted sera were prepared by precipitating at half saturation with ammonium sulphate, re-suspending the washed ppts. in the original vol., and dialyzing against many changes of buffered (pH 7·2) saline for 4 days. The supernatants were also dialyzed for 4 days, concentrated by freeze-drying and made up to their initial vol. with saline.

RESULTS.

A. *Rabbit antisera F and S to egg albumin.*

The antibody contents of the untreated sera were 1·4 mg./ml. for F and 3·7 mg./ml. for S. After they had been extracted the sera were tested over a broad range of antigen concn. and were found to have lost their ability to precipitate. The solutions remained perfectly clear even after 3 days at + 4°. Prolonged centrifugation at 3000 r.p.m. yielded no ppt. The addition of the ether-soluble material at various concn., and dilute aqueous suspension of cephalin or lecithin had no effect. Mixing the extracted serum with an untreated heterologous rabbit antiserum, with 20 per cent bovine albumin, and with horse serum before adding the antigen did not restore precipitation.

Mixtures of the extracted sera and of untreated anti-egg-albumin rabbit serum were prepared at various ratios (from 5 : 1 to 1 : 50), and tested with the previously determined optimal amount of antigen. Although the mixtures required standing over-night in the refrigerator to flocculate, all formed precipitates. As can be seen from Tables I and II the amount of ppt. at a given antigen concn. was constant at different ratios (ranging from 5 : 1 to 1 : 5) of untreated to extracted

serum, so long as the total vol. of antiserum remained the same. Thus, the apparent antibody content of the sera was almost unchanged by lipid extraction.

TABLE I.—*Precipitates Formed by Mixtures of Extracted and Untreated Anti-ovalbumin Serum F in Various Proportions. Ovalbumin added:* 0·064 *mg.*

Extracted serum F (ml.), undiluted.	Untreated serum F (ml.), undiluted.	Ratio, untreated to extracted serum.	Precipitate (mg.).
0	0·6	—	0·96
0·1	0·5	5/1	0·99
0·2	0·4	2/1	1·04
0·3	0·3	1/1	0·99
0·4	0·2	1/2	1·11
0·5	0·1	1/5	0·99
(diluted 1/10)	(diluted 1/100)		
0·5	1·0	1/5	0·117
0·5	0·5	1/10	0·054
0·5	0·25	1/20	0·043
0·5	0·10	1/50	0·026
0·5	0	—	0·005

TABLE II.—*Precipitates Formed by Mixtures of Extracted and Untreated Serum S in Various Proportions.*

Extracted serum S (ml.).	Untreated serum S (ml.).	Ratio, untreated to extracted serum.	Ovalbumin added (mg.).	Precipitate (mg.).
0	0·2	—	0·064	0·80*
0·1	0·10	1/1	,,	0·75
0·06	0·14	2·3/1	,,	0·77
0·04	0·16	4/1	,,	0·74
0	0·3	—	0·064	0·99*
0·2	0·1	1/2	,,	0·97
0	0·25	—	0·056	0·81*
0·2	0·05	1/4	,,	0·83
0·2	0·02	1/10	,,	0·42
0·2	0·01	1/20	,,	0·29

* Calculated from curve, Fig. 1.

The lowest ratio of untreated to extracted serum that would produce the maximum precipitation from a given amount of extracted serum was determined, and proved to be 1 : 5 for serum F and 1 : 4 for serum S (Tables I and II).

The effect of a heterologous precipitating system (horse serum albumin and rabbit antiserum) on the extracted antibody was investigated to determine whether the incomplete antibody would co-precipitate. No increase in the amount of protein precipitated was noted in the presence of the extracted sera.

The quantitative precipitin curve was determined for a 1 : 4 mixture of untreated and extracted serum S and compared to that of an equal amount of un-

treated serum alone (Fig. 1). While the curves coincided in the zone of antibody excess, they differed sharply once the equivalence point was reached. A small excess of antigen, which had little effect on the amount of ppt. obtained from the untreated serum, caused marked inhibition in precipitation from the mixed antisera.

The equivalence point cannot be determined in the usual manner by testing the supernatant fluids with further antigen and antibody, since it might contain non-precipitating antibody. It was assumed that all the antigen was precipitated by mixtures of one part of complete and 4 parts of incomplete antibody up to the point of maximum precipitation; the difference between total precipitate and antigen was called "apparent antibody."

The globulin fraction of the extracted serum was tested with antigen and was

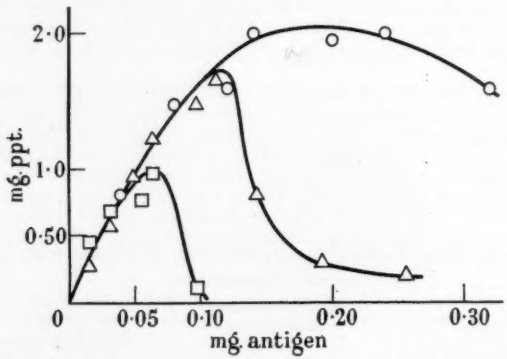

FIG. 1.—Precipitation curves of extracted and untreated anti-egg-albumin rabbit serum.
⊙ untreated serum (vol. 0·5 ml.);
△ mixture of untreated and extracted serum at ratio 1 : 4 (total vol. 0·5 ml.);
⊡ globulin fraction of extracted serum (vol. 0·4 ml. equivalent to 0·28 ml. of undiluted serum).

found to be precipitable. During dialysis 10 ml. of the solution were diluted to 13 ml. The precipitation curve is not smooth (Fig. 1); but if the ordinates and abscissae are multiplied by 5/0·28 to allow for this dilution and for the smaller volume used, the smoothed curve coincides with that obtained with the 1/4 mixture.

The reconstituted extracted serum prepared by mixing equal volumes of the globulin and albumin fractions (kept at room temp. overnight after mixing) retained its ability to precipitate.

Electrophoresis of the serum before and after extraction showed no apparent changes in pattern or mobilities.

B. *Rabbit antiserum to horse-serum-albumin.*

The antibody content of the untreated serum was 6·3 mg./ml. After extraction the serum no longer precipitated with antigen. Prolonged centrifugation in the cold, as well as addition of cephalin, lecithin and heterologous sera, did not produce a ppt.

As with extracted anti-egg-albumin serum, ppts. formed slowly when antigen was added to mixtures of extracted and unextracted serum. The precipitation curve given by a mixture of 5 parts of extracted serum to 1 part of unextracted antiserum (Fig. 2) coincided with the curve given by the unextracted antiserum up to the equilibrium zone and then fell off steeply. The apparent antibody of the extracted serum was therefore the same as the antibody of the extracted serum. With lower ratios of unextracted to extracted antiserum the amount of ppt. was reduced (Table IV). The amounts of ppt. formed with the 1/10 mixture were less than those formed by untreated serum and the 1/5 mixture throughout the zone

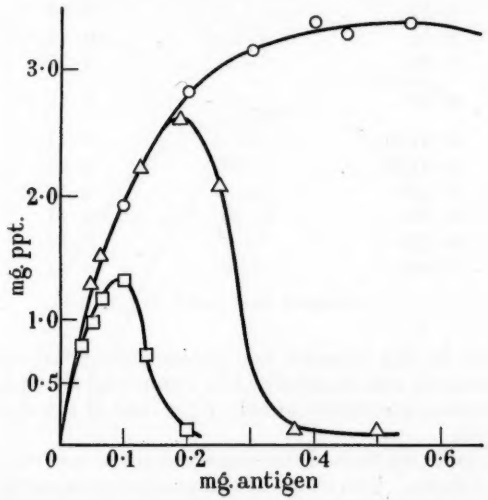

FIG. 2.—Precipitation curves of extracted and untreated anti-horse-serum-albumin rabbit serum.

⊙ untreated serum (vol. 0·5 ml.);
△ mixture of untreated and extracted serum at ratio 1 : 5 (total vol. 0·5 ml.);
⊡ globulin fraction of extracted serum (vol. 0·5 ml. equivalent to 0·27 ml. of undiluted serum).

TABLE III.—*Precipitates Formed by Mixtures of* 0·455 *ml. of Extracted Antiserum with* 0·045 *ml. of Untreated Anti-horse-serum-albumin on Addition of Varying Amounts of Antigen.*

Ratio of untreated to extracted : 1:10.

Horse serum albumin (mg.).	Precipitate (mg.).
0·068	1·29
0·136	1·72
0·204	2·11
0·273	1·72
0·386	0·59
0·546	0·30
0·682	0·16

of antibody excess (Table III); with larger amounts of antigen the curve, like that given by the 1/5 mixture, fell off steeply.

TABLE IV.—*Precipitates Formed by Mixtures of Extracted and Untreated Serum in Various Proportions.*

Extracted serum (ml.).	Untreated serum (ml.).	Ratio untreated to extracted serum.	Horse serum albumin (mg.).	Precipitate (mg.).
0	0·28	—	0·12	1·64*
0·2	0·08	1:2·5	0·12	1·22
0	0·24	—	0·12	1·44*
0·2	0·04	1:5	0·12	1·20
0	0·22	—	0·12	1·36*
0·2	0·02	1:10	0·12	0·98 0·89
0	0·2125	—	0·11	1·34*
0·2	0·0125	1:16	0·11	0·73
0	0·210	—	0·11	1·30*
0·2	0·010	1:20	0·11	0·69
0	0·205	—	0·12	1·29*
0·2	0·005	1:40	0·12	0·18

* Calculated from curve, Fig. 2.

The ppt. formed by egg albumin and antiserum against egg albumin in the presence of the extracted anti-horse-albumin serum slightly exceeded that formed in saline; the difference amounted to only 7 per cent of the antibody of the anti-horse-albumin serum.

The isolated globulin fraction of the extracted antiserum was again found to be precipitable with antigen. The shape of the precipitation curve (Fig. 2) approximates that of the curve obtained with the 1/5 mixture.

c. *Rabbit antiserum to horse globulin.*

This antiserum was prepared by injecting a suspension of the specific ppt. formed by Type I pneumococcal polysaccharide and horse antiserum to Type I pneumococcus. The antibody content was 1·5 mg./ml., precipitable both by salt-dissociated horse antibody and by globulin prepared from the anti-pneumococcal serum by Felton's method.

After lipid extraction of the rabbit antiserum by method B, flocculation with antigen only took place after standing over night at + 4°, as opposed to a few min. at room temp. for the untreated serum. The amount of antibody precipitated and the shape of the precipitin curve, however, remained unchanged by the extraction procedure.

D. *Horse antiserum to pneumococcus Type I.*

After extraction, flocculation of the undiluted serum was delayed from 15 min. to 18 to 24 hr. after antigen addition. A mixture of equal vol. of extracted and untreated sera flocculated in 3 hr. The amount of protein precipitated by antigen was not affected (in fact, appeared slightly increased), but the shape of the

precipitin curve was changed (Fig. 3) in that more antigen was required to produce the same amount of ppt.

Dialysis in the cold against 0·01 M phosphate buffer, pH 5·6, yielded a heavy ppt. which only redissolved partially when readjusted to its former vol., pH and salt concn. Unextracted serum when treated in this fashion produced a much smaller ppt. which redissolved easily in a fraction of the original vol.

The euglobulin fraction prepared in this way from the extracted serum (4·2 per cent protein) was tested with antigen. The amount of precipitable protein was twice that of the whole serum (11 mg./ml.)

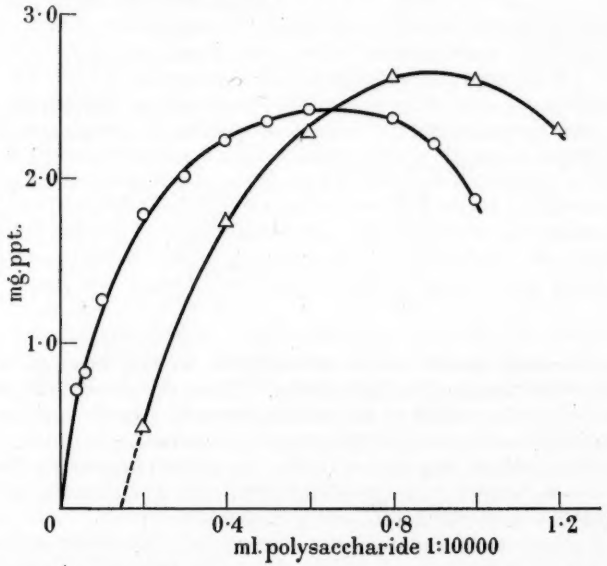

FIG. 3.—Precipitation curves of extracted and unextracted anti-pneumococcal (Type I) horse serum (vol. antiserum : 0·5 ml.).

⊙ unextracted serum.
△ extracted serum.

The pseudoglobulin fraction (2·8 per cent protein) did not precipitate with antigen, even when lecithin was added. When mixed with varying amounts of untreated serum, however, this fraction added protein to the specific ppt. over a broad range of antigen concn. (Table V). When the ratio of extracted pseudoglobulin to untreated serum became sufficiently high (i.e., 5 : 1 by vol. or 2 : 1 by wt. of protein), the precipitation of the untreated serum was inhibited (Table V).

DISCUSSION.

The extraction of lipids resulted in delayed flocculation for an anti-horse-globulin rabbit serum, in complete loss of flocculation for anti-egg-albumin and anti-horse-serum-albumin rabbit sera, and in a change of the precipitin curve of

TABLE V.—*The Effect of Extracted Pseudoglobulin on the Precipitation of Untreated Serum by Type I Polysaccharide.*

Extracted pseudoglobulin (ml.).	Untreated serum (ml.).	Type specific polysaccharide (mg.).	Precipitate (mg.).
	1·0	0·125	5·20
1·0	1·0	0·125	6·50
0·2	0·1	0·01	0·64
0·2	0·1	0·03	0·65
0·2	0·1	0·07	0·64
0·2	0·1	0·09	0·62
0	0·1	0·01	0·36
0	0·1	0·03	0·48
0	0·1	0·07	Inhibition zone of antigen excess
0	0·1	0·09	
(undiluted)	(diluted 1:10)		
0·3	0·6	0·01	105 μg.
0·3	0·3	0·01	38 ,,
0·3	0·1	0·01	27 ,,
0·3	0	0·01	36 ,,
0	0·6	0·01	282 ,,

an anti-pneumococcal horse serum attributable to the incomplete antibodies demonstrated in the pseudoglobulin fraction. Thus, the generalization of Krueger and Heidelberger (1950) based on the investigation of 3 horse antisera concerning the rôle of lipids in immunological reactions does not seem justified.

Any hypothesis about the nature of the incomplete antibodies formed by the alcohol-ether extraction of anti-egg-albumin and anti-horse-serum albumin rabbit sera must take into account the following observations :

1. Loss of ability to flocculate without any loss of apparent antibody protein or of specificity.

2. Apparently complete precipitation of all the antibody from mixtures of extracted antiserum with unextracted antiserum, up to ratios of 4 or 5 parts of extracted to 1 of unextracted in the case of anti-egg-albumin serum, and 5 to 1 in the case of anti-horse-serum-albumin serum.

3. Coincidence of the precipitation curves, given by a mixture of one part of unextracted antiserum with 4 or 5 parts of extracted antiserum, with the curves given by unextracted serum up to the beginning of the equivalence zone.

4. Sharp inhibition in the zone of antigen excess.

5. Recovery of ability to flocculate on fractionation with ammonium sulphate.

The treatment used in extraction of lipids may remove the ability to flocculate with antigen in three ways :

1. The absence of the lipid may itself directly affect the flocculation by increase in solubility.

2. The treatment may distort the antibody molecules either through the direct action of the solvents used, or, indirectly, as a result of removal of lipids, which may be essential for maintaining the configuration of the molecules.

3. Aggregation of the proteins of serum.

These three changes in the antibodies may lead to loss of ability to flocculate either, according to Bordet's theory, through increased solubility of the antibody or, according to the lattice theory, through loss of a valency.

Neuzil (1948) and Tayeau *et al.* (1949) take the first view. This simple explanation disagrees with the claim of Delsal, Perez and Laporte (1950) that equally or more effective removal of lipids by solvents other than ethanol, for example acetone and/or ether, does not affect the rate of flocculation. However, Katsura (1950) did not confirm this claim, as antitoxic serum extracted with acetone did not flocculate with toxin.

However, it is not necessary to postulate that the non-flocculating antibody molecules, *when free*, should be any more soluble than flocculating antibody molecules. According to the Bordet theory, as expounded by Boyd (1946), the insolubility of the antigen-antibody complex is due to occlusion of polar groups between adjacent antibody molecules with consequent loss of attraction for water. Failure to flocculate may be due to some change in the structure of the antibody molecules, as a result of which this occlusion of polar groups does not occur when the antibody molecules combine with antigen. The antibody molecules may be distorted or aggregated with other protein molecules so that they fit less closely and that the polar groups cannot be occluded. Such changes might be reversed when the globulin of the extracted serum was precipitated with ammonium sulphate.

Loss of a valency, demanded by the lattice theory, might be due to distortion of the antibody molecules or to aggregation with obstruction of the approach of antigen to antibody. Katsura (1950), in a paper which appeared since this work was done, reported that extracted diphtheria antitoxin did not flocculate with toxin ; but, if the extracted antitoxin was hydrolysed with pepsin, the ability to flocculate with toxin was restored. This is evidence against distortion of the molecule as a cause of loss of ability to flocculate. Katsura found evidence, in the electrophoresis pattern, of aggregation of the proteins of extracted antitoxic serum and evidence of such aggregations has been found by ultracentrifuge studies (Pedersen, 1945). The restoration of ability to flocculate by hydrolysis with pepsin, and the fact that purified, hydrolysed antitoxin did not lose its ability to flocculate on extraction, strongly suggest that the loss of ability to flocculate is due to aggregation. However, no evidence of aggregation was found in the electrophoresis patterns after extraction of the rabbit anti-egg-albumin and anti-horse-albumin. Dr. C. G. Pope, who had himself observed this effect of hydrolysis, suggested (verbal communication) another explanation : that extraction of lipids exposes a highly soluble part of the antibody molecule ; hydrolysis removes this highly soluble part, leaving a part of the molecule which flocculates more readily than the original antitoxin.

The most remarkable features of the present work are the ability of the extracted rabbit sera to flocculate when mixed with a small amount of unextracted antiserum and the coincidence of the precipitation curves in the zone of antibody excess. According to the lattice theory a molecule of complete antibody combines with two antigen molecules. If one valency is lost, and each antibody molecule can combine with one antigen molecule only, the ratio of antibody to antigen in the compounds formed should be raised. This increase should be found in the precipitates formed on adding antigen to the mixtures of univalent antibody with

a small amount of bivalent antibody; actually it was not found (Fig. 1 and 2). If the loss of a valency were due to aggregation, the apparent molecular weight of the antibody would be increased and the ratio of total precipitate : antigen in the precipitates should be increased still more. Such an increase, attributed to aggregation, was found, for example, by Heidelberger, Krueger and Deutsch (1951) in precipitates formed by antibodies prepared by alcohol precipitation and by salt fractionation of horse antiserum. The coincidence of the curves is, therefore, in favour of the Bordet theory.

But, on either theory, it is difficult to account for the restoration of ability to flocculate by small amounts of untreated antibody. On the Bordet theory the rôle of the untreated antibody would be to fit in with the extracted antibodies and occlude polar groups. On the lattice theory the rôle is to tie the antigen molecules together. On either hypothesis at least one unextracted antibody molecule per antigen molecule would be needed. In the maximum precipitate formed by the mixture of 1 part of unextracted anti-egg-albumin serum with 4 parts of extracted antiserum, the molecular ratio of "apparent" antibody to antigen was 3·7—giving $\frac{3 \cdot 7}{5}$ molecules of unextracted antibody per molecule of antigen. In the maximum precipitate formed by the mixture of 1 part of unextracted anti-serum-albumin serum with 5 parts of extracted antiserum, there were $\frac{5 \cdot 6}{6}$ molecules of unextracted antibody per molecule of antigen. Considerable precipitates were formed in mixtures of extracted antisera with even less unextracted antisera (for example, with a ratio of 1/10, Tables I, II, III and IV); in these precipitates there was considerably less than one unextracted antibody molecule per molecule of antigen. Explanations of the loss of ability to flocculate, based on either theory, present difficulties if it is assumed that the change in antibody molecules is complete. If, however, we suppose that the change is incomplete and consists in a weakening of valency or reduction of the loss of attraction for water when the antibody is combined with antigen, either explanation is possible.

SUMMARY.

Lipid extraction in the cold with ethanol and ether resulted in complete loss of flocculation of rabbit antisera to egg albumin and horse serum albumin, delayed but complete flocculation of rabbit antiserum to horse globulin, and delayed flocculation with minor changes in the precipitin curve of horse antiserum to Type I pneumococcus.

Precipitation of the non-flocculating antibodies did not occur with the addition of the extracted lipids, fresh serum, cephalin or lecithin, or in the presence of a heterologous precipitating antigen-antibody system.

In the presence of small and well-defined amounts of homologous unextracted antiserum and antigen complete precipitation of the non-flocculating antibodies was obtained.

The quantitative relationships between extracted antibody, untreated antibody and antigen have been studied.

The nature of the non-precipitating antibodies is discussed in the light of various hypotheses—changes in solubility, univalence and aggregation.

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CARBOHYDRATE METABOLISM AND CELL DIVISION IN DEVELOPING RED BLOOD CELLS.

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PREVIOUS investigations have shown that the carbohydrate metabolism of the circulating red blood cells of the chicken embryo can be measured in terms of the consumption of oxygen (O'Connor, 1951). In this way it was shown that, during embryonic development, carbohydrate metabolism and the number of dividing cells decreased in such a way as to suggest interdependence. In order to investigate further the relationship between cell division and carbohydrate metabolism in the developing red cells of the chicken embryo, observations have been made on the changes produced in the dividing cells when cellular carbohydrate metabolism is inhibited by fluoride and iodoacetate.

MATERIALS AND METHODS.

In all experiments the blood of embryos of approximately 1·4 mm. eye diameter was used, this stage being reached during the fourth day of incubation. In such embryos the characteristics of the circulating red blood cells are (O'Connor, 1952):

Haemoglobin content	7·5 per cent (wt./vol.).
Cell volume	620 cu.μ.
Dividing cells:	
(*a*) Total	3·8 per cent.
(*b*) Pro-metaphase	2·3 per cent.
(*c*) Ana-telophase	1·5 per cent.
Rate of respiration	0·36 μl. $O_2/10^6$ cells/hr.

In such cells staining reactions for ribonucleic acid are given by the nucleolus and the cytoplasm and the cells show the characteristics of growing cells described by Caspersson (1950).

Cells were removed from the hearts of embryos with a fine pipette and made into suspensions as described previously (O'Connor, 1951). The suspensions contained about 10^5 red cells per c.mm., but the number of immature white cells was not sufficient to affect any of the observations made. Unless otherwise stated all experiments were performed with cells suspended in the following medium: NaCl 0·9 g., KCl 0·02 g., $MgCl_2$ 0·02 g., glucose 0·10 g., water 100 ml., to which was added 10 ml. Sørensen's 0·066 M buffer to produce pH 7·4.

The measurement of respiratory inhibition by fluoride and iodoacetate.—For this purpose a modification of the Cartesian diver micromanometer was used (O'Connor, 1951). In each of a series of experiments divers were set up containing known numbers of red cells from the same cell suspension. To these were added varying concentrations of one of the inhibitors. Decrease in the rate of respiration was measured by comparing results with a control diver containing red cells from the same suspension but without the addition of inhibitor. The comparison was made for the period 1–2 hr. after the addition of inhibitor

and results expressed as a percentage of the control. Using both fluoride and iodoacetate, sufficient experiments were performed to construct curves relating inhibition of respiration to concentration so that the effect of any particular concentration of either inhibitor could be determined by interpolation.

The measurement of the effect of fluoride and iodoacetate on respiratory quotient and aerobic glycolysis.—To make these measurements appropriate modifications were made in the contents of the Cartesian divers (see O'Connor, 1950*a*). To make the measurements of aerobic glycolysis the red cells were suspended in the following medium: NaCl 0·9 g., KCl 0·02 g., $MgCl_2$ 0·02 g., glucose 0·10 g., $NaHCO_3$ 0·20 g., water 100 ml. The gas in the divers was 5 per cent CO_2/95 per cent O_2.

The estimation of the effect of fluoride and iodoacetate on dividing cells.—After measuring the inhibition of respiration it was possible to remove the red cells from the divers and examine the dividing cells microscopically. However, the same results were obtained when

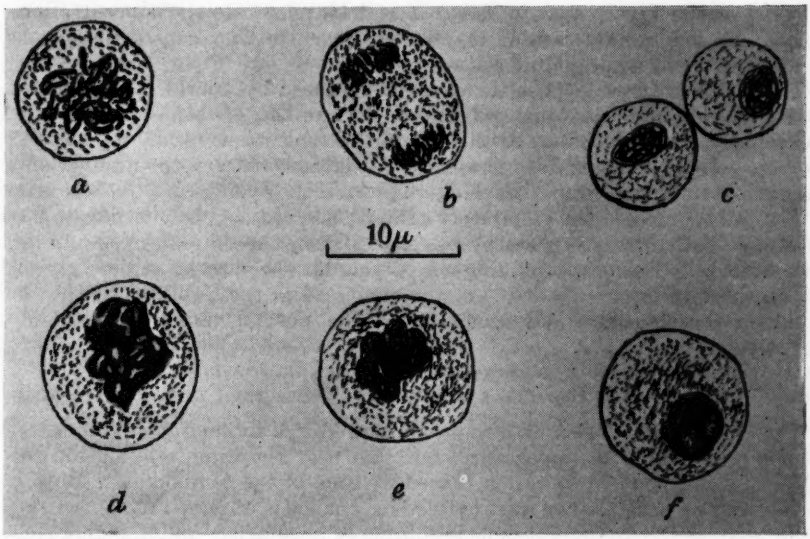

FIGURE.—*Camera lucida* drawings of red blood cells from the 4-day chicken embryo stained with aceto-lacmoid; *a–c*, normal stages of mitosis; *d–f*, the effect of fluoride and iodoacetate. In Tables I and II *d–f* are designated as +, ++ and +++ respectively.

red blood cells were exposed to similar conditions and inhibitor concentrations in small test-tubes. The following procedure was therefore adopted: Dilutions of fluoride and iodoacetate were made in round-bottomed tubes about 10 cm. in length and 1·0 cm. in diameter. Each tube contained about 1·0 ml. fluid. To each tube a drop of a cell suspension was added and the cells were allowed to settle. The fluid in the tubes was then reduced to a layer 2–3 mm. deep and the air replaced by oxygen. The tubes were then corked and incubated at 37° for 90 min., this period being chosen to correspond with the period 1–2 hr. for which the inhibition of respiration was determined. After incubation the cells were stained with aceto-lacmoid as previously described (O'Connor, 1951) and examined microscopically. Abnormal cytological appearances were noted as well as the distribution of cells among various phases of cell division. For the latter purpose the dividing cells in 2000–3000 cells were placed in a pro-metaphase group or in an ana-telophase group in order to distinguish between the stages before and after the movement apart of the daughter chromosomes. The figures obtained were expressed as percentages of the normal values.

RESULTS.

After exposure of the embryonic red blood cells to fluoride and to iodoacetate for 90 min. the cytological changes were similar. With both compounds, in the lower concentrations there was swelling of the chromosomes in metaphase and anaphase ; in higher concentrations the chromosomes were caused to fuse into a structureless mass. In the Figure these changes are represented and compared with normal dividing cells. Similar changes were produced by fluoride and iodoacetate in dividing cells of the mid-brain of the chicken embryo (O'Connor, 1950*b*), and in both tissues the effect resembles that produced by certain mitotic poisons and described as " pycnosis " by Dustin (1947). The extent of the swelling and fusion of chromosomes in the red blood cells has been assessed by standards indicated in the Figure and, in Tables I and II, related to the concentrations of fluoride and iodoacetate causing it. These concentrations caused no change in the appearance of non-dividing cells.

Tables I and II record also the changes produced by fluoride and iodoacetate in the total number of dividing cells and in the numbers of each of the two groups considered. It should be noted that, at all concentrations used, the number of prophase cells was too small to produce a significant effect on the number of cells in the pro-metaphase group. Each figure given is the average of 6–8 observations.

The Tables include the respiratory inhibitions produced by fluoride and iodoacetate. The figures given are interpolations from curves relating inhibition to concentration. The effects of the compounds on the rate of aerobic glycolysis and on the respiratory quotient are not expressed in the Tables. Neither compound, in the concentrations used, altered the normal values of zero and 1·0 respectively.

DISCUSSION.

The swelling and fusion of chromosomes produced by fluoride and iodoacetate are not only similar in appearance, but also bear the same relationship to the degree of inhibition of respiration caused by each of the compounds. With both fluoride and iodoacetate the least cytological change is observed in concentrations which produce the first measurable inhibition of respiration, and in higher concentrations the changes in the chromosomes and the inhibition of respiration increase in a comparable fashion (see Tables I and II). Thus an association is suggested

TABLE I.—*The Effect of Fluoride on the Red Blood Cells of the 4-day Chicken Embryo.*

Concentration, $\times 10^{-3}$ M	1·5	3·0	6·0	12	24
Respiration (per cent of normal)	100	90	80	50	30
Number of dividing cells (per cent normal) :					
(*a*) Total	100	130	140	160	140
(*b*) Pro-metaphase	100	160	180	230	200
(*c*) Ana-telophase	100	100	30	20	18
Effect on chromosomes (estimated as indicated in Fig.)	0	+	++	++	+++

TABLE II.—*The Effect of Iodoacetate on the Red Blood Cells of the 4-day Chicken Embryo.*

Concentration, $\times 10^{-5}$ M	0·17	0·67	2·7	11	43
Respiration (per cent normal)	100	90	80	55	32
Number of dividing cells (per cent normal):					
(*a*) Total	100	78	70	68	60
(*b*) Pro-metaphase	100	90	90	85	100
(*c*) Ana-telophase	100	80	60	20	20
Effect on chromosomes (assessed as indicated in Fig.)	0	+	+	++	+++

between the changes in the chromosomes and the inhibition of respiration. In the embryonic red cells the respiratory quotient is unity and aerobic glycolysis is zero, and these values are not altered by fluoride or by iodoacetate. Respiration, therefore, can be taken as a measure of carbohydrate metabolism, so that the association of the changes produced in the chromosomes with the inhibition of respiration implies a similar relationship to carbohydrate metabolism. A consideration of the mode of action of fluoride and iodoacetate also suggests that the changes in the chromosomes are due to inhibition of carbohydrate metabolism. Although both substances are inhibitors of carbohydrate metabolism they affect different enzyme systems. Hence it may be inferred that the similar effect they produce on the chromosomes of dividing cells is due to the common effect of inhibiting carbohydrate metabolism.

In addition to the changes in the chromosomes, fluoride and iodoacetate produce a decrease in the number of cells in the ana-telophase group of dividing cells, but not in the pro-metaphase group. This decrease in the ana-telophase group can be ascribed to the failure of cells to pass from metaphase to anaphase because the swelling of the chromosomes prevents the daughter chromosomes from moving apart, and is therefore a result of the inhibition of carbohydrate metabolism. There is a considerable degree of correspondence in the case both of fluoride and of iodoacetate, between the inhibition of respiration (which measures carbohydrate metabolism) and the decrease of the number of dividing cells in the ana-telophase group.

Fluoride and iodoacetate differ in their effect on cells in the pro-metaphase group. Fluoride produces an increase of 2–3 times the normal number, while the slight decrease produced by iodoacetate is of doubtful significance. This difference cannot be ascribed to a difference in the penetrating power of the two compounds for, if the effect of 12×10^{-3} M fluoride is compared with the effect of 11×10^{-5} M iodoacetate, it is seen that all the effects, other than that on the number of cells in the pro-metaphase group, are the same. Since both fluoride and iodoacetate prevent cells passing from metaphase to anaphase, it is to be concluded that with fluoride cells continue to enter division, whereas with iodoacetate they do not. It does not appear probable that this failure is due to the inhibition of carbohydrate metabolism produced by iodoacetate, for an equivalent inhibition by fluoride does not prevent cells entering division and causing an increase in the number of cells in the pro-metaphase group. Although the

present experiments are not adequate to suggest the mechanism whereby iodoacetate prevents the entry of embryonic red cells into division, reference may be made to Rapkine's (1931) conclusions that iodoacetate can inhibit cell division directly by the oxidation of –SH groups without necessarily inhibiting carbohydrate metabolism. In addition it can be pointed out that the difference between the effects of fluoride and those of iodoacetate here recorded for the pro-metaphase group of dividing red blood cells in the chicken embryo was not demonstrated in the effect of these substances on dividing cells in cultures of the frontal bone of the 12-day chicken embryo (Hughes, 1950) nor in those of the adult mouse epidermis (Bullough and Johnson, 1951). In these tissues both fluoride and iodoacetate are effective in preventing cells from entering division, and in the mouse epidermis the result is ascribed to the inhibition of carbohydrate metabolism.

Previously it has been shown (O'Connor, 1950*b*) that in the mid-brain of the chicken embryo fluoride and iodoacetate produce cytological changes in dividing cells similar to those produced in embryonic red cells in the present experiments. In the mid-brain, however, the changes were associated with inhibition of aerobic glycolysis but not of respiration, which was unaffected by the concentrations of the inhibitors used. In both tissues, therefore, completion of cell division is prevented when carbohydrate metabolism is inhibited, but in the mid-brain the dependence is on glycolysis, while in embryonic red cells, as shown above, it is on the breakdown of carbohydrate involving the consumption of oxygen. Since carbohydrate metabolism provides energy it is possible to suggest that, although differing in this respect, dividing cells, in both embryonic mid-brain and blood, need access to a source of energy during the actual process of cell division, and this is probably the case in the cultured frontal bone of the chick embryo, because in this tissue Hughes (1950) has shown that fluoride and iodoacetate prevent the completion of cell division after it has begun. In the epidermis of the adult mouse, on the other hand, Bullough and Johnson (1951) have shown that, once begun, cell division is refractory to metabolic inhibitors, including fluoride and iodoacetate; they conclude that the energy required for completion of division is stored in cells before the onset of this process.

If this difference between the metabolic relationships of dividing cells in embryonic and adult tissues were to signify a general difference, this would be of considerable interest. A difference of this kind acquires special significance in view of the similarity said to exist between the metabolism of malignant and embryonic tissues, for it is upon differences between the metabolic mechanisms governing cell division in malignant and normal adult tissues that the possibility might depend of obtaining agents which act specifically on dividing cells in malignant tissue.

SUMMARY.

In dividing red blood cells of the 4-day chicken embryo fluoride and iodoacetate cause chromosomes to swell and cell division is not completed.

These changes are associated with inhibition of cellular carbohydrate metabolism and are ascribed to it.

It is concluded that the embryonic red cells need access to an energy source during the process of cell division and that, in this respect, they resemble dividing

cells in certain other embryonic tissues but differ from those in adult mouse epidermis.

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THE AGGLUTINATION OF SENSITISED RED CELLS BY ANTIBODY TO SERUM, WITH SPECIAL REFERENCE TO NON-SPECIFIC REACTIONS.

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THE purpose of this work was to investigate the degree of species specificity manifested by reactions between red cells coated with antibody, and antisera prepared against various normal sera. These antisera were also treated with the normal sera of various species and the annulling effects of the latter upon the antisera were estimated. For convenience, an immune serum prepared by immunising an animal of species X against serum of an animal of species Y is termed *antiserum X* v. *Y* : species Y serum (or its protein constituents) is spoken of as *corresponding* to that antiserum, while the sera of species other than Y are said to be *non-corresponding*.

The reaction between an antiserum and the corresponding normal serum may be demonstrated by the formation of a visible precipitate : the specificity of such reactions has been fully investigated by Nuttall (1904) and many subsequent workers, and is too well known to require comment. A second method of demonstrating the reaction, depending upon the fixation of complement by the antiserum-serum complex, was investigated by Muir and Martin (1906) who concluded that the specificity was similar to that of the precipitin reaction. A third method of detecting such a reaction depends on the anti-immune-body phenomenon of Bordet (1904), who recorded that when red cells were treated with a haemolytic immune-body (IB) and washed free of the uncombined elements of the immune serum, then the addition of an antiserum corresponding to the serum of the species in which the immune-body was developed protected the sensitised red cells from the lytic action of subsequently added complement ; thus the antiserum acted as an anti-immune-body. This reaction may be represented as follows :

Ox RBC + IB r *v.* ox 56°	W + gp C	→ lysis.		
Ox RBC + IB r *v.* ox 56°	W + gp *v.* r antiserum 56°	W + gp C	→ partial or no lysis.	
Ox RBC + IB r *v.* ox 56°	W + gp normal serum 56°	W + gp C	→ lysis.	

Note.—IB = immune-body. C = complement. RBC = red blood cells freed from native serum. The vertical dotted lines mean a period of incubation at 37°. W indicates that the red cells were washed with saline at this stage. 56° means that the serum was inactivated by previous heating at 56°. r denotes rabbit and gp guinea-pig.

In Bordet's experiments the red cells at the final stage were suspended in inactivated guinea-pig serum, but Muir and Browning (1906) observed a similar protective effect with cells suspended in saline. Moreover, the protective effect of the antiserum was shown to depend not upon interference with the union of immune-body with red cells, but upon its ability to prevent the fixation of complement by the sensitised cells. This property of the antiserum was annulled by the previous addition to it of the corresponding normal serum, or by adding the normal serum to the sensitised red cells which had been treated with anti-immune-body.

$$\text{Ox RBC} + \text{IB r } v.\ \text{ox } 56^\circ \;\Big|^{\,W}_{\,+\ \text{gp } v.\ \text{r antiserum } 56^\circ}\;\Big|^{\,W}_{\,+\ \text{r normal serum } 56^\circ}\;\Big|^{\,W}_{\,+\ \text{gp C}}\;\Big| \rightarrow \text{lysis.}$$

Muir and Browning showed that whereas normal rabbit serum was capable of combining with antiserum guinea-pig *v.* rabbit, and thus of diminishing the protective action of the latter, normal ox or guinea-pig serum had no such effect, an observation which suggests that an antiserum combines with the corresponding normal serum, but not with non-corresponding sera, i.e., that the annulling effect is specific.

Moreschi (1908) observed that the addition of guinea-pig *v.* rabbit antiserum to ox red cells which had been sensitised with haemolytic immune-body derived from the rabbit, and subsequently washed, resulted in agglutination of the cells. That this phenomenon was not recorded earlier may have been due either to variations in the agglutinability of the red cells of individual oxen (*vide infra*), or to the presence of uncombined constituents of the immune-body-containing serum interfering with the combination of antiserum and sensitised red cells. The phenomenon observed by Moreschi provides a fourth method of detecting a serum-antiserum reaction, and may be represented as follows:

$$\text{Ox RBC} + \text{IB r } v.\ \text{ox } 56^\circ \;\Big|^{\,W}_{\,+\ \text{gp } v.\ \text{r antiserum } 56^\circ}\;\Big| \rightarrow \text{agglutination.}$$

$$\text{Ox RBC} + \text{IB r } v.\ \text{ox } 56^\circ \;\Big|^{\,W}_{\,+\ \text{gp normal serum } 56^\circ}\;\Big| \rightarrow \text{no agglutination.}$$

Coombs, Mourant and Race (1945*a*, *b*) demonstrated similarly the occurrence of agglutination when human red cells sensitised with iso-immune-body were treated with an antiserum rabbit *v.* man; they successfully applied the observation to the detection of the Rh antibodies first described by Race (1944) as "incomplete" and by Wiener (1944) as "blocking" antibodies, which alone do not agglutinate red cells suspended in saline.

Boorman, Dodd and Loutit (1946) showed that the red cells in cases of acquired haemolytic anaemia were agglutinated by antiserum rabbit *v.* man, and adduced this as evidence that the red cells were sensitised with an auto-immune-body.

Similar results have been reported by Zoutendyk and Gear (1950) in lupus erythematosus. Thus there is evidence that an antiserum contains antibody capable of uniting with a corresponding immune-body attached to red cells, with consequent agglutination of the latter.

In spite of these recent important observations, reports upon the species-specificity of the reaction are few and the available evidence suggests a low specificity. Thus Simmons (1946) recorded that human red cells sensitised with incomplete Rh (anti-D) antibody of human origin were agglutinated by antisera developed in the rabbit by injecting serum of either man, pig, cat, horse, goat, dog or ox. The effective titres of the various antisera are not stated; therefore it is possible that such agglutination when occurring only with low dilutions of the non-corresponding antisera might be of a non-specific nature, and due to the protein of the antisera producing an effect similar to that of normal human serum (Diamond and Abelson, 1945), bovine albumin (Diamond and Denton, 1945), or gelatin (Fisk and McGee, 1947) upon human cells sensitised with incomplete Rh antibody. Simmons (1946) noted, however, that rabbit *v.* hen antiserum did not agglutinate such sensitised cells, and so it seems likely that his positive results with the other non-corresponding antisera were due to true antigen-antibody reactions. Some of Simmons' antisera were obtained by the method of Proom (1943) for developing antisera to be used in determining the species of tissue extracts and body fluids by the precipitin reaction. Stratton (1950) reported that hen *v.* rabbit antiserum, prepared by Proom's method, agglutinated not only ox red cells sensitised with immune-body rabbit *v.* ox, but also human red cells sensitised with incomplete Rh iso-antibody. Coombs and Mourant (1947) observed that a rabbit *v.* horse antiserum, also prepared by Proom's method, agglutinated " in very low titre " human red cells sensitised with incomplete Rh iso-antibody, while dilutions of 1 in 4 or greater of immune sera from rabbits injected with the typhoid bacillus or pneumococcus did not agglutinate the sensitised human cells. Thus there is evidence, although largely of a qualitative nature, that antisera are capable of taking part in non-specific reactions.

$$\text{RBC of P} + \text{IB Q } v.\ \text{P } 56^{\circ} \left|\begin{array}{l} \text{W} \\ + \text{ antiserum S } v.\ \text{T} \end{array}\right| \rightarrow \text{agglutination.}$$

(P, Q, S, T represent animals of different species.)

In view of the generally accepted high degree of species specificity of reactions between whole serum and the corresponding antiserum, it seems surprising that considerable non-specificity should be exhibited in reactions between antiserum and immune-body, and the experiments which follow were undertaken to investigate the specificity of such reactions. For this purpose antisera were prepared by immunising rabbits with human and with horse serum, and by immunising hens and goats with rabbit serum. Red cells sensitised with immune-body from man, horse and rabbit were used for testing the antisera. It should be added that to begin with precautions were taken to remove by absorption, from the various antisera and normal sera, agglutinins for the red cells concerned, so that agglutination of the sensitised cells following the addition of an antiserum can be accepted as indicating an antigen-antibody reaction between the antiserum and immune-body. As there is considerable individual variation in the specificity

of antisera, those investigated in the present work were examined also by precipitin reactions with the normal sera of various species; the results provide a comparison for the reactions of the antisera with sensitised red cells. In the account which follows the term *immune-body* (IB) is reserved for the antibody produced by the injection into an animal of heterologous red cells, which in suitable dilutions is capable of uniting with red cells of the species injected without agglutinating them. Two antibodies were used to sensitise human red cells—an Rh iso-antibody and an auto-antibody from a case of acquired haemolytic anaemia. For convenience, the term immune-body, although perhaps not strictly applicable, is used also to describe these two antibodies. All sera were inactivated by heating at 56° before use.

MATERIALS AND METHODS.

Preparation of Antisera.

Twelve antisera were examined in all, and as the investigation concerns their specificity, the methods of preparation are given in detail.

Antiserum rabbit v. *man* (r *v.* m): Five antisera (Nos. 1–5). No. 1 was prepared by Proom's method, two simultaneous intramuscular injections of 5 ml. of alum-precipitated human serum being given, and these were repeated after an interval of 4 weeks, the rabbit being bled 14 days later. For Nos. 2 and 3 human serum was incorporated with the adjuvant mixture described by Freund and McDermott (1942). A single subcutaneous injection was given, containing 0·25 ml. human serum, 0·25 g. emulsifying agent ("Euserine," Herts Pharmaceuticals, Ltd.), and 1 mg. of heat-killed dried tubercle bacilli—(I am indebted to Dr. A. E. Francis of the Wellcome Research Laboratories for this material)—suspended in 0·5 ml. liquid paraffin. After four months each rabbit received 2 injections of 1 ml. human serum alone, the first intraperitoneally and the second intravenously 2 days later; after an interval of 4 weeks the rabbits received 5–7 injections of 1 ml. human serum alone at 1–2-day intervals, the first being intraperitoneal and the rest intravenous; the rabbits were bled 14 days after the last injection. Nos. 4 and 5 were prepared as Nos. 2 and 3, except that the initial injection of serum-adjuvant emulsion was given intraperitoneally.

Antiserum rabbit v. *horse* (r *v.* eq): Four antisera (Nos. 1–4) were prepared—No. 1 by Proom's method, Nos. 2–4 similarly, but followed 6 weeks later by 1 ml. horse serum intraperitoneally and 1 ml. intravenously on the following day; the rabbits were bled 10 days after the last injection.

Antiserum hen v. *rabbit* (hen *v.* r): Of two hens immunised by Proom's method, one showed only a weak antibody response, and no antibodies could be detected in the serum of the other.

Antiserum goat v. *rabbit* (g *v.* r): Two goats, one adult and one four months old, were immunised with rabbit serum by the same method as for rabbit *v.* horse antisera Nos. 2–4, but double quantities were injected throughout. The antisera (Nos. 1 and 2) were active only in low dilution; they were concentrated to about 8 times the original strength by freezing, allowing to thaw without shaking, and collecting the concentrated deeper portion.

Sensitised red cell suspensions.

Blood was stored in sterile citrate-saline solution at 4° and was used within 48 hr. of collection. Portions were removed as required, the red cells washed 3 times with saline, and a 3 per cent suspension prepared. This suspension was treated for 1 hr. at 37° with a suitable amount of immune-body, and the cells were again washed thrice with saline and a 3 per cent suspension reconstituted in saline; this was used only on the day of preparation, and before use a portion was incubated for 1 hr. at 37° and the absence of agglutination confirmed. Also, the agglutinability of the suspension was examined with doubling dilutions of an appropriate antiserum. Suitable antisera were kept for testing each species of sensitised red cells, and by their use any gross variation in the strength of sensitisation was detected, insensitive or supersensitive suspensions being discarded.

Immune-body.

Group 0 human red cells of probable Rh genotype CDe/cde were sensitised with a human serum containing incomplete Rh (anti-D) antibody. The concentration used was 8 times the minimum required to render the cells agglutinable by an antiserum rabbit *v.* man. Human red cells were also used from a case of haemolytic anaemia ; these cells were strongly sensitised with auto-immune-body, and merely required to be thoroughly washed with saline to provide a suspension of sensitised cells. Sheep red cells were sensitised with approximately 2 m.h.d. of an immune-body horse *v.* sheep. (Supplied by Burroughs Wellcome & Co. for use in the Wassermann reaction.)

Ox cells were sensitised with an immune-body rabbit *v.* ox prepared by immunising a rabbit with 6 intraperitoneal injections of washed ox red cells at fortnightly intervals and bleeding 14 days after the last injection. Several rabbits were immunised thus, and in using their sera two difficulties were encountered. Firstly, the titre of agglutinin in the heated immune serum (r *v.* ox) was almost as great as the titre of immune-body rendering the cells agglutinable by hen or goat *v.* rabbit antiserum ; this necessitated using the immune sera in high dilutions, a procedure likely to effect weak sensitisation. The following is an example :

Titre of agglutinin of IB r *v.* ox 56° for ox RBC = 1/2000 ;
titre of IB r *v.* ox 56° rendering ox cells agglutinable by hen or goat *v.* r antiserum = 1/10,000 ;
dilution of IB used = 1/5000.

The second difficulty concerned variations in the behaviour of different specimens of ox red cells ; while an immune serum showed practically the same haemolytic titre (with excess of guinea-pig complement) for all specimens, the titre of agglutinin for ox cells alone and of antibody effecting sensitisation of ox cells to the antiserum hen or goat *v.* rabbit, although running parallel with each other (the sensitising titre being the higher), showed considerable variation with cells from different individuals. It was found advisable to use cells which were agglutinated and sensitised to the action of antiserum by high dilutions of immune serum. Such variations in ox cells have been described also by Gleeson-White, Heard, Mynors and Coombs (1950).

Titration of antisera.

To determine the capacity of an antiserum to react with an immune-body, one-drop amounts of doubling dilutions of the antiserum were prepared in a series of 7 × 50 mm. tubes with a Pasteur pipette. To minimise dilution errors the pipette after every alternate transfer was rinsed successively in a considerable volume of saline in two containers and the saline was changed frequently ; this method proved sufficiently accurate for the purpose. To each dilution of antiserum a drop was added of a suspension of red cells sensitised with immune-body, and after incubation for 1 hr. at 37° the cells were examined by a hand-lens, and the titre of agglutination observed more accurately by microscopy. Each series of tubes included a control containing saline in place of antiserum dilution, and a duplicate series was also set up containing normal in place of sensitised red cells. These controls were always negative and are not included in the tables of results.

Precipitin tests were performed in 5 × 70 mm. tubes with 0·15 ml. of a suitable dilution (usually 1 in 5) of antiserum, and 0·15 ml. of doubling dilutions of normal serum from 1 in 4 upward. Usually the ring method was employed and the presence or absence of turbidity observed after 1 hr. at room temperature. Culbertson (1932) regarded this technique as providing an indication, not of the strength of a precipitating antiserum, but of the amount of reacting precipitinogen in the normal serum. Whether or not this view is correct, the technique, under properly controlled conditions, is of well established value for determining the species of protein solutions (Boyd, 1947 ; Glaister, 1950), and its application in the present work is based on the assumption that tests suitable for deciding the species of an antigen are suited also to estimate the species specificity of an antibody. Precipitin tests were also carried out in which increasing dilutions of normal serum were mixed with a constant dilution of antiserum. Where it was necessary to determine the optimally reacting proportions of serum and antiserum the method of Dean and Webb (1926) was employed. Pre-immunisation specimens of serum were obtained from all the animals in which antisera were developed, and were shown to be devoid of ability to agglutinate any of the suspensions

of sensitised red cells employed or to produce turbidity when tested with any of the normal sera used as precipitinogens.

RESULTS.

I. *Specific and Non-specific Agglutination of Sensitised Red Cells Effected by Antisera.*

The twelve antisera were tested with each of the three species of sensitised red cells; the results (Table I) show that an antiserum, in general, agglutinates

TABLE I.—*The Agglutinating Titres of Antisera for Red Cells Sensitised with Immune-bodies Developed in Three Different Species.*

Antiserum.	Human RBC + human Rh IB.	Sheep RBC + IB horse *v.* sheep.	Ox RBC + IB rabbit *v.* ox.
r *v.* m 1	8192	1024	2
,, 2	1280	640	—
,, 3	2560	1280	—
,, 4	320	80	—
,, 5	1280	160	4
r *v.* eq. 1	16	512	2
,, 2	32	2560	—
,, 3	8	1280	—
,, 4	4	160	—
g *v.* r 1	64	16	128
,, 2	16	256	128
hen *v.* r	32	2	32

In this and other tables, r denotes rabbit, m man, eq horse and g goat. IB = immune body. All sera inactivated by heating at 56°.

The figures represent the titres of the antisera, i.e., the highest dilutions of antisera causing agglutination.

— = No agglutination with any dilution of antiserum.

Controls.—Each titration included a tube containing sensitised red cell suspension, and saline in place of antiserum dilution. All titrations were duplicated, using normal in place of sensitised red cells. These controls were always negative and are not included in the tables.

most powerfully red cells sensitised with immune-body derived from the species against which the antiserum has been developed. It is clear that the antisera may react also with non-corresponding immune-bodies; e.g., rabbit *v.* man antiserum agglutinates sheep red cells combined with immune-body horse *v.* sheep, while goat *v.* rabbit antiserum agglutinates all three species of sensitised cells. The human red cells used in this experiment were sensitised *in vitro* with incomplete Rh antibody. When auto-sensitised human red cells were used the non-corresponding antisera in general yielded higher titres; in particular the titres of antisera rabbit *v.* horse 1–4 were respectively 32, 128, 128 and 16. No significant reaction occurred between antisera produced in the rabbit and the immune-body rabbit *v.* ox, a result which was, of course, to be expected. The antiserum hen *v.* rabbit reacted only in low dilutions, and was not used in the experiments which follow. In none of the tests was the prozone effect sufficiently pronounced to cause complete absence of agglutination with low dilutions of antiserum.

II. *Specific and Non-specific Reactions between Normal Serum and Antiserum.*

The above experiments demonstrated that all the antisera tested react with a non-corresponding immune-body attached to red cells provided that the antiserum and the immune-body have not been developed in the same species. In such reactions the antigen consists presumably of the immune-body globulin of the sensitising serum, and it might therefore be supposed that the antiserum would be capable of reacting with the globulin fraction of normal serum of the same species as the immune-body. Accordingly, precipitin tests were performed with the antisera, using normal corresponding and non-corresponding sera as precipitinogens. The results of tests by the ring method are shown in Table II.

TABLE II.—*Precipitin Reactions. The Highest Dilution of Normal Sera of Various Species with which a 1 in 5 Dilution of the Antisera Produced a Definite Turbidity in the Ring Test.*

Normal sera.	Antisera rabbit *v.* man.					Antisera rabbit *v.* horse.				Antisera goat *v.* rabbit.	
	1	2	3	4	5	1	2	3	4	1	2
Man	8,000	16,000	128,000	8,000	64,000	32	128	8,000	—	—	—
Horse	256	256	32,000	128	8,000	16,000	16,000	16,000	8,000	—	—
Rabbit	—	—	—	—	—	—	—	—	—	512	512
Ox	2,000	4,000	16,000	256	8,000	1,000	256	8,000	512	—	—
Pig	1,000	4,000	16,000	64	8,000	1,000	..	2,000	—	—	—
Sheep	—	2,000	4,000	16	1,000	128	..	512	128	—	—
Goat	—	1,000	8,000	16	2,000	256	..	512	128	—	—
Hen	16	—	—	—	—	—	4	8	8	—	—

— = No turbidity observed.
.. = Tests not performed owing to insufficient antiserum.
The figures represent the reciprocals of the highest dilutions of normal sera with which turbidity developed in the region of the interface with the antiserum.

It will be noticed that in general antisera rabbit *v.* man and rabbit *v.* horse show a low degree of specificity in that they react with high dilutions of various non-corresponding normal mammalian sera. It must be noted, however, that turbidity usually developed more rapidly in reactions between antisera and the corresponding normal sera than in the non-specific reactions with non-corresponding sera. Thus if the results were read after 10 min. instead of 1 hr., the non-specific reactions were much weaker than those shown in Table II, whereas the specific reactions were reduced only slightly, e.g., in 1 hr. the antiserum rabbit *v.* man 5 showed precipitation with human serum diluted 1 in 64,000 and with horse serum 1 in 8,000; when the time was reduced to 10 min. the highest dilutions with which a positive reaction was seen were respectively 1 in 8,000 and 1 in 64. Further, when some of the tests were repeated with mixtures of the reagents instead of layering, the time of reading was again seen to be important, a long period favouring the development of non-specific reactions. Also, a greater amount of precipitate formed in the specific than in the non-specific reactions. Determination of the optimally reacting proportions of antiserum and serum did not assist in eliciting differences between specific and non-specific reactions, since the proportion was often the same for both corresponding and non-corresponding normal sera. None of the antisera reacted strongly with normal hen serum, the only non-mammalian serum used, and the relatively weak goat *v.* rabbit antisera did not show non-specific reactions.

III. *The Effect of Various Normal Sera and Immune-bodies upon the Capacity of Antisera to React with the Corresponding Immune-bodies.*

The above results showed that an antiserum may react with a non-corresponding immune-body or normal serum, but they do not indicate whether any considerable proportion of the antibody in the antiserum participates in such non-specific reactions. In order to decide this the antiserum has been treated with (*a*) various normal sera and (*b*) various immune-bodies, and subsequently tested with the corresponding immune-body.

(*a*) Equal volumes of the antiserum and a normal serum (or dilutions of these) were mixed and incubated for 1 hr. at 37°; the mixture was then tested in doubling dilutions with the corresponding immune-body attached to the appropriate suspension of sensitised red cells; in this manner the antiserum was tested after the addition of normal sera of various species. The stronger antisera were used in an initial dilution of 1 in 10, the weaker ones undiluted. The results obtained with an antiserum rabbit *v.* man are given in Table III, and show that

TABLE III.—*Agglutinating Titre for Red Cells Sensitised with Rh Iso-antibody of Antiserum Rabbit* v. *Man* 2 *after Admixture with Normal Serum of* 8 *Species.*

Nil (control) . .	1280	Goat . . .	2560
Man . . .	<40	Rabbit . . .	2560
Ox . . .	640	Horse . . .	1280
Pig . . .	2560	Hen . . .	2560
Sheep . . .	1280		

strong inhibition of the antiserum was effected by treatment with the corresponding (human) normal serum, and that no inhibition followed treatment with any non-corresponding serum. The minor variations in titre following treatment of the antiserum with non-corresponding normal sera were shown by repeated experiments to be inherent in the method of preparing doubling dilutions. A modification which eliminated such inaccuracies, and also enabled the higher dilutions of antiserum to be treated with greater amounts of normal serum, consisted in preparing the doubling dilutions of antiserum in large quantities and distributing these in 1 drop amounts: 1 drop of 1 in 10 dilution of a normal serum was then added to each tube, followed 1 hr. later by 1 drop of the sensitised red cell suspension.

Tests of all the antisera are summarised in Table IV. In every case addition of non-corresponding normal serum did not noticeably reduce the capacity of

TABLE IV.—*The Effect on the Reaction between Corresponding Antisera and Sensitised Red Cells of Prior Treatment of the Antisera with Normal Sera of Various Species.*

Antiserum.	Species of normal serum added to antiserum.			
	Man.	Horse.	Rabbit.	Others.*
Rabbit *v.* man . .	+	—	—	—
Rabbit *v.* horse . .	—	+	—	—
Goat *v.* rabbit . .	—	—	+	—
Hen *v.* rabbit . .	—	—	+	—

\+ Denotes complete inhibition of agglutination.
— ,, absence of ,, ,,
* Includes ox, pig, sheep, goat and hen serum.

an antiserum to react with its corresponding immune-body, whereas the addition of corresponding normal serum to an antiserum annulled the reaction.

(*b*) The above conclusion seems surprising in view of the demonstration that many antisera are capable of reacting, some in high dilutions, with non-corresponding immune-bodies. It was therefore thought possible that an immune-body attached to red cells might react more strongly with a non-corresponding antiserum than does normal serum. Accordingly, sensitised red cells, after being well washed with saline and packed, were added to an antiserum which agglutinated them although not corresponding to the species from which the immune-body was derived ; after 1 hr. at 37° the antiserum was removed by centrifuging and the treatment with a fresh portion of the same sensitised red cells was repeated several times until the antiserum failed to agglutinate them. The treated antiserum was subsequently tested with its corresponding immune-body. An example of the results is shown in Table V (A). Here it is seen that the agglu-

TABLE V.—*The Effect on the Agglutination Titre of Corresponding Antiserum for Sensitised Red Cells of Previous Treatment of the Antiserum with Suspensions of Red Cells Sensitised with an Immune-body not Corresponding to the Antiserum.*

A. Antiserum goat *v.* rabbit 2.		Agglutination titre of antiserum for ox RBC + IB r *v.* ox.
Untreated		128
Treated with	Human RBC + human Rh IB	64
	Sheep RBC + IB horse *v.* sheep	64
	Ox RBC + IB rabbit *v.* ox (control)	8

B. Antiserum rabbit *v.* man 5.		Agglutination titre of antiserum for human RBC + Rh iso-antibody.
Untreated		512
Treated with	Sheep RBC + IB horse *v.* sheep	256
	Human RBC + human Rh IB (control)	8

tination titre of the antiserum goat *v.* rabbit for red cells sensitised with immune-body from the rabbit (IB r *v.* ox) suffered no reduction as a result of previous thorough treatment with a non-corresponding immune-body (human and horse) combined with the homologous red cells. On the other hand, of course, similar treatment with the corresponding immune-body (IB r *v.* ox) practically removed the agglutinating power of the antiserum. Table V (B) shows the results of similar experiments upon the antiserum rabbit *v.* man 5. The antisera goat *v.* rabbit 1 and rabbit *v.* man 2 were also investigated in this manner.

In no case was an appreciable loss of capacity to react with the corresponding immune-body found to result from treatment of an antiserum with a non-corresponding immune-body attached to red cells. Hence there is no evidence that a greater proportion of the anti-globulin antibody of an antiserum participates in the reaction with a non-corresponding immune-body than with a non-corresponding normal serum.

IV. *The Amount of Corresponding Normal Serum which on Addition to an Antiserum Effects Complete Inhibition of the Reaction of the Latter with its Corresponding Immune-body.*

The result established in Section III, that treatment of an antiserum with the corresponding normal serum can inhibit completely the capacity of the antiserum to react with corresponding immune-body, had to be examined quantitatively. Antiserum and corresponding normal serum were mixed in various proportions, and after incubation for 1 hr. at 37° a series of one-drop amounts of doubling dilutions was prepared from each mixture. To each of the dilutions one drop was added of the appropriate suspension of sensitised red cells; after a further hour at 37° the presence or absence of agglutination was noted. Table VI shows the results

TABLE VI.—*Agglutinating Action for Iso-sensitised Human Red Cells of Antiserum Rabbit* v. *Man* 4 *after Admixture with Various Quantities of Human Normal Serum.*

Dilution of normal human serum added to an equal volume of undiluted antiserum.	Titre of treated antiserum for iso-sensitised human red cells.*
1 in 5	<3
1 in 20	<3
1 in 60	<3
1 in 200	3
1 in 500	6
1 in 2,000	24
Titre of untreated antiserum	768

* Each stock mixture contained equal volumes of undiluted antiserum and the specified dilution of normal serum; after preparation of serial dilutions and addition of red cell suspension, the dilutions of antiserum in the successive tubes of each series are 1 in 3, 1 in 6, 1 in 12, etc.

of such an experiment, in which mixtures of the antiserum rabbit *v.* man 4 with normal human serum were tested with human red cells sensitised with Rh antibody. It is seen that the addition of a relatively low concentration (1 in 200 to 1 in 2,000) of the normal serum produces strong inhibition, while higher concentrations are completely inhibitory. All the other antisera were tested similarly, and it was found that the greatest dilution of corresponding normal serum which effected complete inhibition of an equal volume of undiluted antiserum lay between 1 in 60 and 1 in 20, while the dilution which reduced the titre of the antiserum to less than one-tenth lay between 1 in 200 and 1 in 2,000. In view of the observation in Section III that the capacity of an antiserum to react with the corresponding immune-body was not diminished by relatively large amounts of non-corresponding normal sera, the present experiment emphasises the high specificity of inhibition by the corresponding normal serum.

V. *The Effect on the Specific Precipitin of an Antiserum of Treatment with Non-corresponding Normal Sera.*

Three antisera after treatment with non-corresponding normal sera were tested with the corresponding normal serum as precipitinogen. The normal sera used for treating each antiserum were those with which the antiserum was known to react strongly (Section II). The optimally reacting proportion of each normal serum, as determined by the method of Dean and Webb (1926), was mixed with a suitable quantity of a 1 in 5 dilution of the antiserum; after standing for 3 hr.

at room temperature and overnight at 4° the precipitate was centrifuged out, and the supernatant fluid removed and tested by the ring method with doubling dilutions of the corresponding normal serum. In a control series the antiserum was treated with the corresponding normal serum. A modification of the technique consisted in treating the antiserum twice with the optimal proportion of normal serum and centrifuging after each treatment, the supernatant being examined subsequently as described. The results of testing an antiserum rabbit *v.* man are shown in Table VII, from which it is seen that treatment of the anti-

TABLE VII.—*The Effect on the Precipitin Reaction between Antiserum and Corresponding Normal Serum of Prior Treatment of the Antiserum with Non-corresponding Normal Serum.*

Antiserum rabbit *v.* man 3 treated with normal serum of—	Highest dilution of normal human serum showing precipitation with the treated antiserum.	
	Antiserum treated once.	Antiserum treated twice.
Man (control)	512	256
Horse	16,000	16,000
Ox	32,000	32,000

Highest dilution of normal human serum showing precipitation with untreated antiserum = 32,000.

serum with non-corresponding normal sera causes no reduction of the specific precipitin, while similar treatment with the corresponding normal serum causes a very marked reduction. The same result was obtained with the other two antisera, rabbit *v.* man 5 and rabbit *v.* horse 3, which were similarly examined.

VI. *Evidence that Non-specific Reactions are due to a Special Moiety of Antibody Contained in the Antisera.*

The question arises whether the reactions of certain antisera with constituents of non-corresponding sera, as evidenced by precipitation of normal serum or agglutination of red cells sensitised with an immune-body, are in fact due to a non-specific portion of antibody in the antisera. Experiments carried out in parallel with those in Table V (A) showed that treatment of the antiserum goat *v.* rabbit 2 with a non-corresponding immune-body (IB horse *v.* sheep), combined with the homologous red cells, removes from the antiserum the capacity to react with this immune-body, the agglutination titre of the antiserum being reduced from 128 to <8; whereas the titre of the treated serum for the corresponding immune-body (IB rabbit *v.* ox) was unchanged. This observation strongly suggests the existence of such a non-specific antibody fraction. Therefore the two following experiments were performed:

(*a*) The antiserum rabbit *v.* man 2, initially diluted 1 in 2·5, was treated with an equal volume of a 1 in 2·5 dilution of normal serum of various species as described above, and subsequently tested with a suspension of sheep red cells sensitised with immune-body horse *v.* sheep. The results (Table VIII) show that almost complete removal of agglutinating power is effected by the corresponding (human) serum, and by the non-corresponding serum of the same species as that containing the immune-body, namely horse serum, while the other non-corresponding normal sera are without inhibitory effect. All the agglutinating combinations demonstrated in Section I were investigated from this point of

TABLE VIII.—*The Effect on the Reaction between an Antiserum and Non-corresponding Immune-body of Prior Treatment of the Former with Normal Serum of Various Species.*

Antiserum rabbit *v.* man 2 treated with normal serum of the following species—	Agglutination titre for sheep RBC sensitised with IB horse *v.* sheep.
Man	10
Horse	<10
Ox	640
Pig	640
Sheep	640
Goat	320
Rabbit	1280
Hen	320
Titre of untreated antiserum	640

TABLE IX.—*The Effect on the Reactions between Antisera and Non-corresponding Immune-bodies of Prior Treatment of the Antisera with the Normal Serum of Various Species.*

Antisera.	Normal sera used to treat antisera.					Sensitised red cells used to test treated antisera.
	Human.	Horse.	Rabbit.	Pig.	Others.*	
Rabbit *v.* man 1–5.	+	+	—	—	—	Sheep red cells sensitised with IB horse *v.* sheep.
Goat *v.* rabbit 1 & 2	—	+	+	—	—	
Rabbit *v.* horse 1–4	+	+	—	—	—	Human red cells sensitised with Rh iso-immune-body.
Goat *v.* rabbit 1 & 2	+	p	+	p	—	

\+ = Complete inhibition of agglutination.
— = No inhibition ,,
p = Partial inhibition ,,
* Includes ox, sheep, goat and hen serum.

view and the results, summarised in Table IX, show that complete removal of agglutinating power was not only brought about by treatment of an antiserum with the corresponding normal serum, but also was always effected by treatment with the non-corresponding normal serum of the same species as that containing the immune-body. Further, in the latter case, as in the former, quantitative experiments similar to those described in Section IV showed that inhibition was effected by small quantities of serum. Treatment with other non-corresponding normal sera was generally without effect, although partial inhibition was sometimes observed, this being confirmed by repeated tests with different samples of these sera.

In Section III it was shown that the reaction between an antiserum and its corresponding immune-body is not appreciably inhibited by previous treatment of the antiserum with a non-corresponding normal serum, and this is the case even although the latter two reagents produce precipitation. The present experiment shows that treatment of an antiserum (e.g., rabbit *v.* man) with non-corresponding normal serum of a species which precipitates it (horse) does inhibit the capacity of the antiserum to react with an immune-body developed in the latter species (horse). Therefore it must be concluded that a large fraction of the anti-immune-body component of the antiserum possesses a high degree of specificity, while the fraction which confers the ability to react with a non-corresponding immune-body is so small that its removal does not appreciably diminish the capacity of the antiserum to react with its corresponding immune-body.

(*b*) Precipitin tests showed that certain antisera react with high dilutions of

particular species of non-corresponding normal serum (Section II). Such an antiserum (rabbit *v.* horse 3) was treated with the optimal proportion of the appropriate non-corresponding normal serum (human or ox), and was subsequently tested by the ring method with these normal sera. As controls, tests were performed in parallel with the corresponding normal serum (horse). The results (Table X) show that the reactions with both non-corresponding normal

TABLE X.—*The Effect on the Precipitin Reaction between Antiserum and Non-corresponding Normal Serum of Prior Treatment of the Antiserum with the Optimal Proportion of Non-corresponding Normal Sera.*

Antiserum rabbit *v.* horse 3 treated with the normal serum of—	Highest dilution of normal serum yielding a precipitate with the treated antiserum.		
	Human serum.	Ox serum.	Horse serum.
Horse (control)	128	128	128
Man	128	128	8,000
Ox	128	8	8,000
Highest dilution of normal serum yielding a precipitate with untreated antiserum	16,000	4,000	32,000

sera are greatly reduced by previous treatment of the antiserum with either of these sera (human or ox)—a result in striking contrast to the observation that the capacity of an antiserum to react with its corresponding normal serum is not appreciably diminished by treatment with non-corresponding normal sera (Section V). Similar tests with antisera rabbit *v.* man 3 and 5 led to the same results: therefore it follows that the non-specific precipitation reactions of an antiserum must be attributed to only a small fraction of its total precipitin content.

DISCUSSION.

The object of this work was primarily to investigate the degree of species specificity manifested when antisera prepared against various sera react with immune-bodies separated from other serum constituents through being attached to red cells. Thus the latter, in the form of washed sensitised cells, constituted the test object on which the activity of the antisera could be assessed by the occurrence of agglutination. Here the antigen presumably consists exclusively of that portion of the serum globulin constituting the immune-body which has been fixed by the red cells; it was thought that this reaction might exhibit a higher degree of specificity than the precipitin reaction between antiserum and whole serum, in which several antigen-antibody systems are concerned. However, the antisera tested were shown to react, sometimes in high dilutions, with immune-bodies derived from animals of species phylogenetically distant from that against which the antiserum was developed. The antisera were also investigated by precipitin tests, using the normal serum of various species as precipitinogens. Here too the results suggested a low degree of specificity, many of the antisera reacting with high dilutions of non-corresponding normal sera.

It has long been accepted from the work of Uhlenhuth (1905) and others that the species specificity of an antiserum is determined partly by the phylogenetic relationship between the species in which it is developed and the species of the serum used to stimulate its development. Thus of two antisera developed in rabbits, one by injecting an avian serum, the other by injecting a mammalian serum, the former is usually of low specificity, since it reacts strongly with the

serum of many avian species, whereas the latter usually reacts strongly only with the corresponding serum and those of closely related species. In the present work antisera developed in mammals by injecting mammalian serum frequently exhibited strong reactions with the immune-bodies or normal sera of mammalian species phylogenetically remote from the corresponding species (Table I). Thus antiserum rabbit *v.* man reacted with immune-body developed in the horse and with normal horse serum ; antiserum rabbit *v.* horse reacted with immune-body derived from human serum and with normal human serum ; antiserum goat *v.* rabbit reacted with immune-bodies, but not with normal serum, from both man and horse.

On the analogy of the standard methods of estimating bacterial agglutinins, haemagglutinins and haemolysins, the strength of the reaction between antiserum and immune-body was assessed by determining the highest dilution of antiserum which reacted with a constant quantity of antigen, and the observation that high dilutions of an antiserum effected agglutination of red cells sensitised with an immune-body of species not corresponding to that antiserum is regarded as reliable quantitative evidence of low specificity of the antiserum. In precipitin reactions, however, a large amount of antiserum is required to produce a visible precipitate ; therefore such tests were carried out usually by determining the highest dilution of normal serum which reacted visibly with a constant low dilution of antiserum. As has been emphasised, the results of precipitin reactions depend largely upon the length of time between setting up the test and reading the result, a long period favouring the development of reactions between antiserum and non-corresponding normal sera. Further, the method does not take into sufficient account the amount of precipitate formed, which is always much less with non-corresponding than with corresponding sera. For these reasons the observation that an antiserum reacts with high dilutions of a non-corresponding normal serum (Table II) is considered to be a less reliable quantitative indication of non-specificity than that afforded by the reaction between antiserum and non-corresponding immune-body.

Although the antisera exhibited a low degree of specificity in their reactions with immune-bodies, it was shown that the addition of non-corresponding normal sera of various species, even in large quantities, to an antiserum did not diminish the capacity of the latter to react with the corresponding immune-body (Table IV). The question arose whether the antiserum might react more avidly with a non-corresponding immune-body attached to red cells than with normal serum of the same species as the immune-body. However, no evidence was obtained in support of this, since the capacity of the antiserum to react with the corresponding immune-body was not diminished by repeated treatment of the antiserum with large quantities of red cells sensitised with an immune-body not corresponding to the antiserum, but with which the antiserum reacted strongly (Table V).

The reaction of a non-corresponding immune-body with an antiserum was shown to be inhibited by prior addition to the latter of a relatively small amount of either the corresponding normal serum, or of normal serum of the same species as the immune-body ; other non-corresponding normal sera were usually without effect, but sometimes caused partial inhibition (Table IX). From these observations it is concluded that even though an antiserum reacts in high dilutions with a non-corresponding immune-body, such a reaction is attributable to only a small fraction of the anti-immune-body contained in the antiserum, while a large part of the anti-immune-body is of a highly specific nature. The effects

of various normal sera on the precipitin reactions of the antisera with corresponding and non-corresponding normal sera suggest that a similar conclusion applies also to the specificity of these reactions, namely that the precipitin is in large part specific, non-specific reactions being attributable to a small fraction. This result agrees with the work of Landsteiner and van der Scheer (1936), who performed adsorption tests on precipitating antisera active against azoproteins, and concluded that such an antiserum contains antibody fractions of different specificities.

Some of the antisera were developed according to the adjuvant technique of Freund and McDermott (1942), by which it was hoped that a single injection might yield a potent and specific antiserum; this hope was not realised, and although a short series of further injections of normal serum alone resulted in antisera of high titre, as has been found by Emerson, Franklin and Lowell (1951), they were of low specificity. Since Proom's method (1943) or the modification of Wootton (1950) produces potent antisera in a high proportion of animals, the procedure of Freund and McDermott (1942) does not seem to justify the time consumed by it.

The well-established fact that an antiserum is annulled by treatment with the corresponding normal serum was observed not only in precipitin reactions with whole serum, but also in agglutinating reactions with red cells coated with immune-body. In the case of the former reaction the inhibition effected by prior treatment with a large quantity, i.e., the optimal amount, of the corresponding normal serum, although strong, was not complete (Table VII), presumably because an antiserum contains several precipitins, each reacting with a precipitinogen present in the corresponding normal serum. Thus the addition of the optimal amount of the corresponding normal serum to an antiserum cannot be expected to furnish a mixture in which the components of each precipitin-precipitinogen system are present in optimal proportions. On the other hand, the addition of a very small quantity of the corresponding normal serum completely annulled the capacity of an antiserum to react with the corresponding immune-body attached to red cells (Table VI). It is suggested that the latter observation might be applied practically for the identification of the species of protein-containing fluid. Thus if the fluid under investigation was likely to be of species H, it would be added to an antiserum prepared against serum of species H, and the treated antiserum would be tested subsequently with a suspension of red cells sensitised with immune-body derived from an animal of species H. Non-agglutination of the sensitised cells would indicate that the protein in the fluid was of species H, whereas their agglutination would show that the protein was of a species other than H. The experiments described above suggest that such a test would be more highly specific than the direct precipitin reaction commonly employed; further work is in progress to ascertain its suitability for medico-legal purposes.

SUMMARY.

Antisera developed against the sera of three mammalian species were tested for specificity (*a*) by their capacity to agglutinate suspensions of red cells coated with various immune-bodies, and (*b*) by the strength of their precipitin reactions with various normal sera. Strong non-specific reactions were encountered, i.e., an antiserum reacts with immune-body or normal serum of non-corresponding

species. Although strong, these non-specific reactions are attributable to a relatively small fraction of the antibody contained in an antiserum, the antibody being, for most part, highly specific.

Prolonged treatment of an antiserum with the optimally reacting amount (i.e., that producing most pronounced precipitation) of the corresponding normal serum does not entirely remove the precipitin contained in an antiserum. By contrast, treatment with the corresponding normal serum, even in high dilution, effects rapid and complete inhibition of the capacity of an antiserum to react with the corresponding immune-body attached to red cells. By this means a method of determining the species of a normal serum is provided which surpasses in specificity the direct precipitin reaction between antiserum and normal serum at present in general use. Furthermore, this procedure follows the classical method of determining the strength of an antigen-antibody reaction by allowing the antiserum to be used in serial dilutions with a constant amount of antigen, a technique not applicable to the precipitin reaction in which the antiserum can be used only in low dilutions.

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ACUTE PULMONARY OEDEMA AND HISTAMINE.

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SEVERAL mechanisms are certainly involved in the production of pulmonary oedema and, experimentally as well as clinically, a great number of factors may initiate this syndrome (Luisada, 1950). Among the chemical factors suspected of taking part in the pathogenesis of pulmonary oedema and perhaps representing a common feature of different types of pulmonary oedema, histamine has for long attracted the attention of workers in this field. Luisada (1950) reports that more than 20 years ago he and his co-workers thought that histamine or acetylcholine produced in the lung led to oedema of that organ, but apparently could not obtain certain proof of this. With the development of specific antihistamine substances, indirect evidence of the rôle of histamine in pulmonary oedema accumulated. At various times Halpern and his co-workers (Halpern and Cruchaud, 1947*a*, *b*, 1948 ; Halpern, Hamburger and Cruchaud, 1948 ; Halpern, Cruchaud and Valade, 1949 ; Halpern and Roux, 1949 ; Vermeil, Halpern and Cruchaud, 1949 ; Cruchaud and Vermeil, 1950), as well as Reuse (1948), have presented evidence of the protective activity of the antihistaminic substance Phenergan against experimental pulmonary oedema provoked in different animal species by various procedures such as intravenous injection of adrenaline or chloropicrin, inhalation of phosgene, or intracisternal injection of a mixture of fibrinogen and thrombin. The protective activity of the antihistaminic agent was attributed by the French workers mainly to its property of preventing increase of cellular and capillary permeability.

On the other hand, Stone and Loew (1949) were unable to protect rabbits against adrenaline-induced pulmonary oedema with Phenergan. In Winter's experiments (1949) Phenergan likewise failed to protect rats or guinea-pigs against pulmonary oedema induced by ammonium chloride or guinea-pigs against pulmonary oedema elicited by adrenaline. MacKay, Jordan and MacKay (1949) also found that Phenergan was without effect on ammonium pulmonary oedema in guinea-pigs, and more recently Cameron and Sheikh (1951) observed only slight, if any, protective activity of antihistaminic drugs against pulmonary oedema induced in rats by ammonium chloride. In view of these conflicting results, I decided to investigate directly the behaviour of the pulmonary histamine in acute pulmonary oedema induced by ammonium chloride in the rat. This type of pulmonary oedema has commonly been classified as neurogenic and adrenergic (Koenig and Koenig, 1949*a*, *b* ; Cameron and Sheikh, 1951 ; MacKay *et al.*, 1949).

METHODS.

Twenty-four male hooded rats which were about the same age when the experiment started were at each stage of the experiment divided into two equal groups of similar weights. One group was injected intraperitoneally with 0·2–0·3 ml. of a 1·5 per cent solution of nembutal per 100 g. body wt. Under this moderate

anaesthesia the rats were bled from the exposed common carotid artery into ice-cooled centrifuge tubes, to which 1 per cent heparin was added, so as to make its final concentration ≡ 1 mg./ml. The three lobes of the right lung were carefully freed of non-lobar bronchi or vessels and weighed separately to the nearest mg. The rats of the other group were injected intraperitoneally with 0·8 ml. of a 6 per cent ammonium chloride solution per 100 g. body wt. (Cameron and Sheikh, 1951). Between 12–25 min. after the injection, when the rats were moribund, they were bled in the same way as the nembutal rats. The three lobes of the right lung were tied off nearest to the pulmonary parenchyma with silk thread and weighed separately. The middle and lower lobes were then sliced with scissors on filter-paper, drained by gentle squeezing between filter-paper and re-weighed. The different lobes were extracted for histamine according to Feldberg and Kellaway's method (1937). In this way the oedematous upper lobes were extracted entire, while the other oedematous lobes were drained of fluid first. In a few cases the histamine content of the expressed oedema fluid was estimated directly.

The haematocrit value of all blood samples was determined in the usual manner. Seven samples of each rat group were extracted for histamine by Code's method (1937).

Furthermore, in five rats of each group a portion of bronchial tissue, consisting of the bronchial stumps of the right side and the main bronchi of the left side, was obtained in the following way : The trachea was cut just above the bifurcation, and as much as possible of the main bronchi of the left side was stripped of pulmonary parenchyma with pincers. After draining between filter-paper this bronchial tissue was weighed and extracted for histamine in the same manner as the lobes.

All histamine determinations were performed on the atropinized guinea-pig's ileum suspended in a 15 c.c. bath at 35° perfused with oxygenated Mg-free Tyrode. A solution of histamine acid phosphate served as reference solution ; all histamine values are expressed in terms of histamine base. The assay was only begun when the gut responded to 0·01 μg. histamine or less ; usually 0·005 μg. histamine caused the gut to contract by about 3–5 mm. The method had an error of not more than 10 per cent.

RESULTS.

Amount of oedema and haematocrit.

In Table I the individual weights of the three lobes of the rat groups are given in terms of mg./100 g. body wt. The ratios of middle/upper and lower/middle lobe in both groups are about the same, so that the oedema fluid was equally distributed over the three lobes of the right lung. It will be seen that the average weights of the oedematous lobes were each about twice that of the normal lobes. The increase in weight is substantially due to the oedema fluid, because after slicing and draining the oedematous lobes on filter-paper, their mean weight was equal to that of the normal lung (see Table II).

The haematocrit increased from an average value of 45·7 in the nembutal rats to one of 60·3 in the rats with pulmonary oedema, i.e., it increased by 32 per cent on the average.

Histamine content of normal and oedematous lung.

In Table III the histamine content of the three lobes of both groups is given in terms of μg./g. tissue. The values for the oedematous upper lobes are those

TABLE I.—*Weights (mg./100 g. body wt.) of the Three Lobes of the Right Lung and Haematocrit of (N) Rats Bled under Slight Nembutal Narcosis, (O) Rats Bled before Dying of Ammonium Pulmonary Oedema.*

Group.	Rat No.	Body weight. (g.)	Upper lobe. (I.)	Middle lobe. (II.)	Lower lobe. (III.)	Haematocrit.
N	1	125	72	84	163	45·5
	2	128	72	84	170	47·5
	3	133	71	75	158	38·5
	4	131	67	73	171	37·5
	5	143	68	77	154	42·0
	6	165	72	76	153	46·0
	7	180	75	61	150	46·5
	8	211	61	69	137	50·5
	9	345	44	56	107	47·0
	10	338	44	50	111	48·5
	11	341	39	42	106	49·0
	12	372	39	44	101	47·5
		Mean	60·3	65·9	140·1	45·7
		Ratios : II/I, 1·09 ; III/II, 2·32.				
O	1	134	135	168	314	50·0
	2	145	145	151	309	61·0
	3	165	142	154	297	56·5
	4	192	115	115	252	67·0
	5	190	205	200	383	60·0
	6	201	130	164	328	60·5
	7	128	137	148	322	60·5
	8	300	102	108	268	63·0
	9	312	94	95	228	58·0
	10	312	100	106	262	62·0
	11	342	97	100	248	64·5
	12	315	80	83	151	61·0
		Mean	123·5	132·7	280·1	60·3
		Ratios : II/I, 1·08 ; III/II, 2·28.				

determined on the tissue extracted with the oedema fluid ; the mean value for this lobe was then doubled to account for the oedema, which was shown above the amount to 50 per cent of the lobe weight. The histamine values of middle and lower lobes are those determined on the drained tissue. It will be seen that the histamine content of each of the oedematous lung lobes was significantly lower than that of the corresponding normal lobes. In those cases in which the histamine content of the expressed oedema fluid was estimated directly it was found not to exceed 0·01 μg./ml.

The histamine content of the lung of the older rats tended to be higher than that of the younger ones, a fact which has also been observed in guinea-pigs by

Trethewie (1947). The lower lobes seemed to yield more histamine than the middle and upper ones, but on statistical analysis this difference was found to be not significant with this number of animals (P = 0·1 — 0·2), nor was the difference between the middle and upper lobes significant (P = 0·4 — 0·5).

TABLE II.—*Effect of Removing Extracellular Fluid by Slicing and Draining on Weights (mg./100 g. body wt.) of Right Middle and Lower Lobes of Oedema Lung.*

Rat No.	With extracellular fluid.		Without extracellular fluid.	
	Middle lobe.	Lower lobe.	Middle lobe.	Lower lobe.
1	168	314	104	205
2	151	309	74	164
3	154	297	75	150
4	115	252	70	166
5	200	383	76	181
6	164	328	71	158
7	148	322	75	160
8	108	268	51	140
9	95	228	55	132
10	106	262	50	132
11	100	248	50	129
12	83	151	46	83
Mean	132·7	280·1	66·4	148·3

Histamine content of main bronchi and whole blood.

The histamine content of the main bronchi, which was determined in 5 rats of each group, apparently remained unchanged in the oedema rats. These figures also clearly indicate that the bronchial tissue of rats contains much more histamine than the lobes. On the other hand, the histamine content of arterial whole blood as determined in 7 rats of each group using Code's method (1937) was higher in the oedema rats (P <·01).

DISCUSSION.

When acute pulmonary oedema is induced in rats by the intraperitoneal injection of ammonium chloride, a depletion of pulmonary histamine occurs. The oedema fluid amounted to 50 per cent of the weight of the oedematous lung, and the uncorrected histamine content of the upper lobe, which was extracted with the oedema fluid, was about half that of the middle lobe extracted after draining off the fluid. Since the histamine content of neither normal upper and middle lobes nor oedematous upper lobe after correction for oedema and middle lobe after draining the oedema differed significantly, it follows that released pulmonary histamine does not enter the oedema fluid in appreciable amounts. The released histamine seems to be carried to the blood stream, where it accounts for the increased histamine content of arterial blood. The question to what extent histamine derived from the lung contributed to the excess of blood histamine found in the oedema rats remains to be elucidated. In Table IV are given the mean histamine values (μg./g.) for the three lobes of seven rats of each group in

which the blood histamine was estimated. The mean haematocrit value of the seven nembutal rats was 44·1 per cent, that of the oedema rats 61·4 per cent; hence the mean red cell fractional volume increased by 40 per cent. On the other hand,

TABLE III.—*Histamine Content of the Three Lobes of the Right Lung, Main Bronchi (μg./g. tissue) and Whole Blood (μg./ml.) of (N) Rats Bled under Slight Nembutal Narcosis, (O) Rats Bled before Dying of Ammonium Pulmonary Oedema.*

Group.	Body weight. (g.)	Upper lobe. (I.)	Middle lobe. (II.)	Lower lobe. (III.)	Main bronchi. (IV.)	Whole blood (V.)
N	125	1·8	2·0	2·2	—	—
	128	1·8	1·8	2·0	—	—
	133	1·0	1·6	1·8	—	0·05
	131	3·2	4·8	6·5	—	0·05
	143	1·7	1·8	2·3	9·5	0·048
	165	0·8	1·8	2·4	—	0·06
	180	1·2	1·6	2·6	—	0·05
	211	1·6	1·8	1·8	—	0·062
	345	2·0	2·8	3·3	12·0	—
	338	4·5	5·5	6·0	12·0	—
	341	4·0	4·5	7·5	11·0	—
	372	8·0	9·5	12·5	22·0	0·06
	Means	2·63	3·29	4·24	13·3	0·054
O	134	0·8*	1·2†	1·9†	—	—
	145	0·25	0·6	0·9	—	0·1
	165	0·5	1·1	1·2	—	0·1
	192	0·25	0·5	0·6	8·5	0·08
	190	0·25	0·45	0·5	8·5	0·085
	201	0·6	1·5	1·6	14·8	—
	128	0·3	0·65	0·85	—	—
	300	0·25	0·7	2·0	—	0·1
	312	0·9	2·2	2·8	16·8	0·09
	312	0·3	0·9	1·3	—	—
	342	0·8	1·8	2·2	18·0	0·18
	315	1·5	1·8	2·4	—	—
	Means	0·558 1·116‡	1·117	1·52	13·3	0·105
	t-tests§	P<·01	P = ·01	P<·02	P>·9	P<·01

* Extracted with oedema.
† Extracted after draining oedema.
‡ Corrected for oedema.
§ *t*-test between corresponding columns.

the mean histamine content of blood increased from 0·54 μg./ml. in the nembutal rats to 0·105 μg./ml. in the oedema rats; i.e., the blood histamine increased by 94 per cent. Since the haematocrit increased by 40 per cent and since the red cells contain very little, if any, histamine, 40 per cent of the 94 per cent increase in blood

histamine of the oedema rats may be simply due to the haemo-concentration. In normal rats of similar weights the weight of the whole lung without non-lobar bronchi was found to be 0·5 g./100 g. body wt. On this basis, and assuming a histamine value for the whole lung without bronchi to be midway between that of the right middle and right lower lobes, a total lung histamine (μg./100 g. body wt.) of 1·85 and 0·63 μg. respectively for nembutal and oedema rats is obtained ; thus about 1·2 μg. histamine was released from the lung. Assuming further a total blood volume of 7 ml./100 g. body wt. and an even distribution of the released pulmonary histamine in the blood, and taking into account a 40 per cent haemo-concentration, the blood of the rats with pulmonary oedema should have shown a histamine content of 0·054 + 0·021 + 0·17 = 0·245 μg./ml. Since on the average

TABLE IV.—*Average Amount of Histamine (μg./g. Lobe of the Right Lung) in 7 Rats of each Group.*

Rat group	Nembutal.			Oedema.		
Lobes	Right upper.	Right middle.	Right lower.	Right upper.	Right middle.	Right lower.
	2·5	3·1	4·3	0·9	1·0	1·5
	≡ 1·85 μg. histamine/100 g. body wt.			≡ 0·63 μg. histamine/100 g. body wt.		

only 0·105 μg./ml. was recovered, part of the released histamine must either have been destroyed in the body during the development of the pulmonary oedema or have escaped into the extravascular space. Some such assumptions seem necessary, particularly because other tissues besides the lung also probably released histamine under the influence of ammonium ion, an effect which has been clearly demonstrated on isolated diaphragm strips by Schild (1949).

Different workers have shown that pulmonary oedema caused by ammonium ion is adrenergic in origin (MacKay *et al.*, 1949 ; Cameron and Sheikh, 1951). Koch and Szerb (1950), furthermore, found that adrenaline would liberate histamine from the isolated rat lung ; and Garan (1938) showed that inhalation of ammonia vapours by the isolated lung would cause broncho-constriction and the appearance of histamine in the perfusate. My findings are direct evidence that histamine depletion of the lung is a feature of acute pulmonary oedema, and they may explain the beneficial effects of antihistaminic drugs reported by French workers (Halpern and Cruchaud, 1947*a*, *b* ; Reuse, 1948). They do not, however, offer any insight into the more intricate mechanism of which histamine depletion appears to be a part, or to answer the question why the comparatively minute quantities of histamine present in the rat lung and the still smaller ones liberated from it in pulmonary oedema should be effective in this syndrome when, as is known, the rat is extremely insensitive to exogenous histamine. It is reasonable however to postulate that the rat is much more sensitive to released endogenous histamine.

SUMMARY.

When acute pulmonary oedema is induced in rats by intraperitoneal injection of NH_4Cl a marked fall of the histamine of the lung tissue itself occurs. The released histamine does not enter the pulmonary oedema fluid in appreciable

amounts, but seems to account for the increase in arterial blood histamine. The main bronchi of the normal rat lung contain 2 to 4 times as much histamine as the lobes, and their histamine content remains unchanged in this type of pulmonary oedema.

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EXPERIMENTAL PRODUCTION OF GINGIVAL HYPERPLASIA IN FERRETS GIVEN "EPANUTIN" (SODIUM DIPHENYL HYDANTOINATE).

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SODIUM diphenyl hydantoinate ("dilantin sodium" or "epanutin") was successfully introduced by Merritt and Putnam (1938*a*, *b*) for treatment of convulsive disorders such as epilepsy. In man, however, these and other authors noted the occurrence of minor toxic symptoms in about 5 per cent of epileptics under epanutin therapy, and some 15 per cent showed more serious reactions in the form of dermatitis and purpura. In addition, from 38–57 per cent of epanutin-treated subjects have been reported as suffering from a peculiarly severe form of gingival hyperplasia which is unresponsive to conservative or local surgical treatment other than tooth extraction, unless the drug is also withheld.

Preliminary experiments on the ferret indicated that in this species similar gingival abnormalities could be induced with ease by epanutin administration in the food (King and Gimson, 1947), and the present paper describes an extension of such studies directed toward the histopathology of the epanutin gum disease. It was also noted that the lesions bore a striking naked-eye resemblance to gingival hyperplasia previously induced in ferrets by urea in the diet or drinking water (King, 1945–6, unpublished). The possible implications of this observation were to some extent enhanced by the fact that the epanutin compound contains an urea radical, and preliminary comparisons between the gum lesions resulting from the use of these two substances have therefore been included here.

MATERIAL AND METHODS.

A total of 69 ferrets has so far been used, each animal receiving the following basal diet daily: Bread, 60–70 g.; whole milk powder, 3·5 g.; baker's yeast, 2·0 g.; salt mixture (McCollum 185), 1·0 g.; vitamin A (1000 i.u.) and vitamin D (100 i.u.) in peanut oil, 2 ml., all mixed and moistened with water. Experimental supplements included short lengths of rib-bone with some attached muscle given with the food to induce gnawing, about 15 g.; epanutin in the diet as a suspension of 8 mg. in 8 ml. distilled water or intramuscularly as a finely-dispersed suspension of 16 mg. in 8 ml. distilled water at 60°; and urea as a solution of 2 mg. in 2 ml. glass-distilled water or in much greater quantity in the earlier studies. The epanutin was in the form of sodium 5:5 diphenyl hydantoinate (Parke, Davis & Co.). The urea was obtained from two different sources, a commercial product (not AnalaR) of British Drug Houses Ltd., and a more purified compound prepared in our laboratories from human urine.

Following formol fixation, serial paraffin sections (5–7μ in thickness) were made of the interdental gum and of the upper carnassial teeth of each animal; in some cases the teeth of the lower carnassial and fourth premolar and upper third premolar and incisor regions were also sectioned. Decalcification of teeth was effected by 2 per cent nitric acid. Staining methods included Ehrlich's haematoxylin and eosin, Weigert's iron haematoxylin and van Gieson, triple stains of Mallory (modified) and of Masson, and Laidlaw's silver technique.

GROSS FINDINGS.

Series 1 (*Table I*).—All of the 7 epanutin animals deficient in dietary bone showed gingival hyperplasia of some degree, but the characteristic pedunculated multilobular growths did not appear until the fourteenth week or later (Figs. 1

TABLE I (Series 1).—*Macroscopic Effects of Epanutin, given in the Food of Ferrets, with and without Dietary Bone. Age at Beginning, 12 weeks.*

Number of animals.	Experimental daily supplements. Epanutin.	Bone.	Total experimental period (weeks).	Gum lesions observed.
1	8 mg. for first 8 weeks, 16 mg. for next 6 weeks	Throughout	14	None.
1	Ditto	None	14	Pedicles.
5	8 mg. for first 8 weeks, 16 mg. for next 12 weeks	,,	20	,,
2	Ditto	Throughout	20	None.
1	8 mg. for first 8 weeks, 16 mg. for next 21 weeks	For first 21 weeks only	29	Forming pedicles.
1	Ditto	For last 8 weeks only	29	None.
2	None	Throughout	20	,,

DESCRIPTION OF PLATES.

FIG. 1.—*In vivo* photograph (× 5) of upper and lower premolar and canine regions of one side of ferret given basal diet (no bone) + 8–16 mg. epanutin daily in food for 21 weeks (Table I). Note gingival hyperplasia with strawberry-like lobulated appearance.

FIG. 2.—Post-mortem photograph (× 5) of same animal's gingivae as in Fig. 1, following continued administration of 16 mg. epanutin daily in food, but with addition of dietary bone for next 8 weeks (Table I). Note disappearance of previous gum lesions.

FIG. 3.—Post-mortem photograph (× 3) of upper premolar, canine and incisor regions of ferret given basal diet (no bone) + 8–16 mg. epanutin daily in food for 20 weeks (Table I). Note severe lobular hyperplasia of gum covering premolars and causing displacement of incisors.

FIG. 4.—Post-mortem photograph (× 3) of similar regions of ferret given same basal diet and epanutin dosage for 20 weeks but with added dietary bone throughout experimental period (Table I). Note normal alignment of incisor teeth and absence of gingival abnormalities.

FIG. 5.—Post-mortem photograph (× 4) of upper premolar and canine regions of ferret receiving basal diet (no bone) + 48 mg. epanutin weekly in food for 43 weeks; dietary bone was given for first 15 weeks only (Table II). Note characteristic gingival hyperplasia.

FIG. 6.—Post-mortem photograph (× 4) of upper premolar and canine regions of ferret given same diet and epanutin dosage as that illustrated in Fig. 5, but drug given intramuscularly (Table II). Note characteristic but slightly less severe gum lesions.

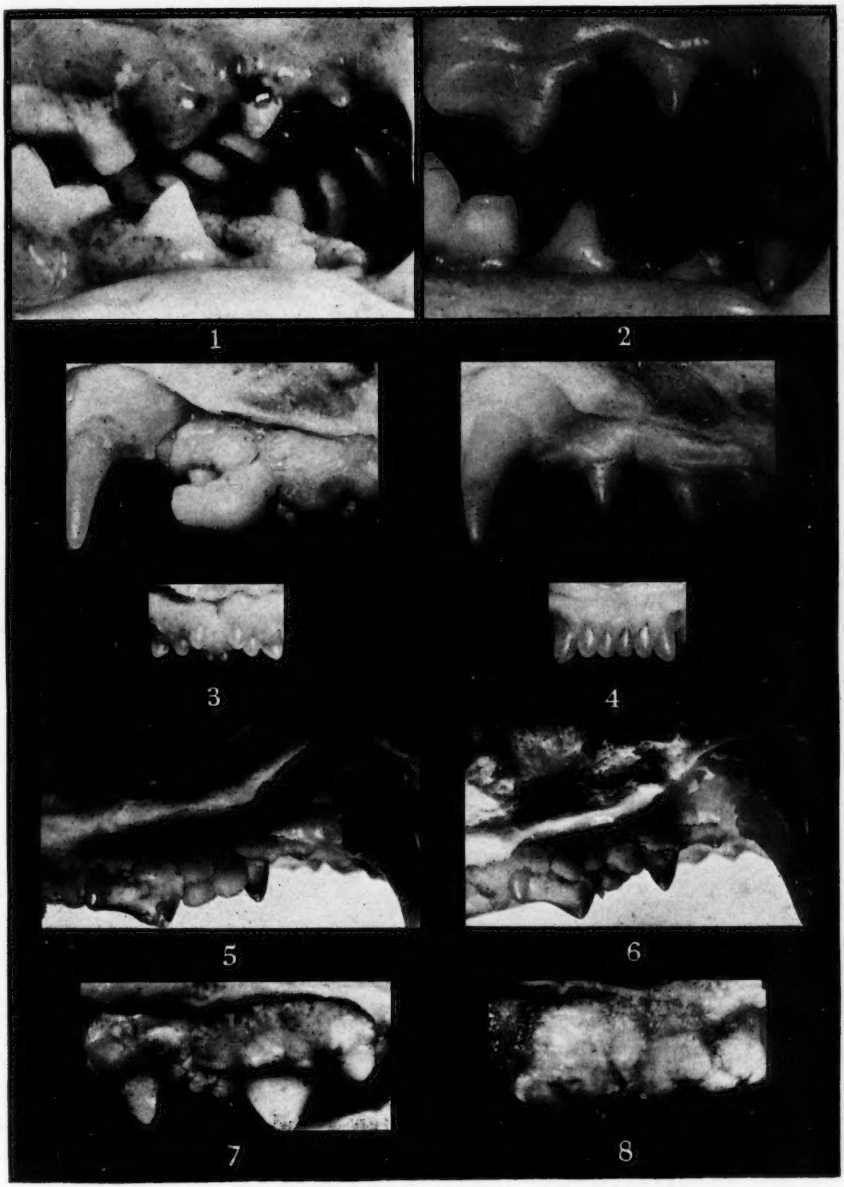
FIG. 7.—Post-mortem photograph (× 5) of upper premolar regions of ferret given 2 mg. urea (B.D.H.) weekly in basal diet for 22 weeks (Table III). Note somewhat lobulated but more diffuse gingival hyperplasia.

FIG. 8.—Post-mortem photograph (× 5) of upper premolar regions of ferret receiving 5 g. urea (B.D.H.) daily for 12 weeks. Extensive hyperplasia of gum conceals all but the tip of one tooth.

FIG. 9.—Photomicrograph (× 90) of labial aspect of upper 4th premolar region of ferret receiving dietary bone and epanutin for 22 weeks. Note hyperplasia of gingival epithelium, and vaso-dilatation and perivascular fibroblast proliferation in corium. Ehrlich's haematoxylin and eosin stain.

FIG. 10.—Photomicrograph (× 90) of labial aspect of upper 3rd incisor of ferret given dietary epanutin (no bone) for 22 weeks, showing beginning of pedicle (P). Laidlaw's silver and neutral red stain.

FIG. 11.—Photomicrograph (× 48) of labial aspect of ferret's upper 4th premolar region showing a later phase of epanutin disease, with the fibrous-cored pedicle (P) rising externally to the original gum crest (G) and calculus (CL). Modified Picro-Mallory stain.



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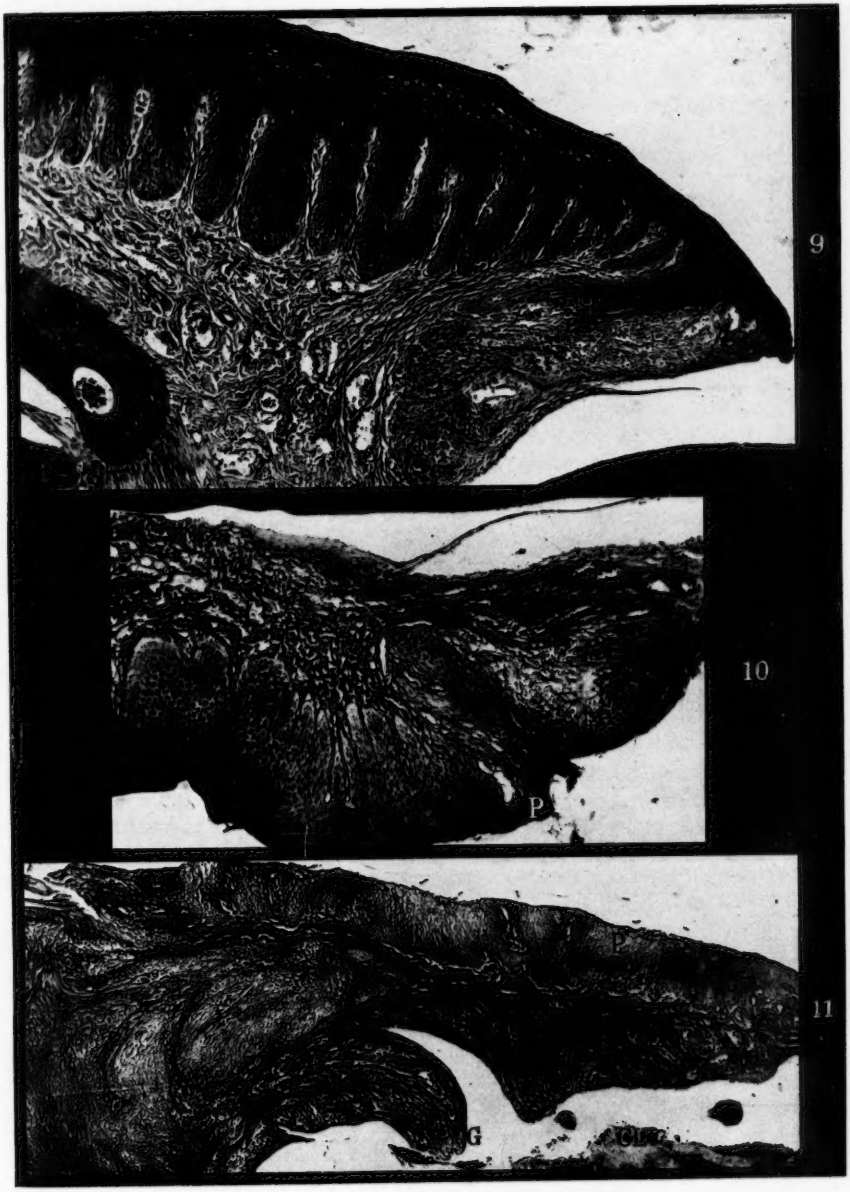

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and 3). The 3 epanutin ferrets receiving bone throughout (Fig. 4), 1 epanutin animal given bone for the last 8 weeks of the 29-week period, and the 2 bone controls exhibited no detectable parodontal abnormalities. The first notable changes in the bone-deficient epanutin ferrets were injection and swelling of the gum in the carnassial regions, the tissues assuming a red-mottled, strawberry-like appearance (Fig. 1); at this and more advanced stages a few weeks of dietary bone served to bring the gingivae back to clinical normality in spite of continued administration of the drug (Fig. 2). As the disease progressed the mottled coloration of the gum tended to disperse and the tissue appeared somewhat ischaemic but, with further elevation of the pedunculate growths which covered the tooth crowns and pushed the teeth out of alignment (Fig. 3), inflammation of the gum due to periodical masticatory and other trauma gave rise to secondary injection and haemorrhage. A definite association was apparent not only between absence of dietary bone and susceptibility to the epanutin disease, but also between the site and severity of the lesions and those of dental calculus, the deposition of which was in turn related to proximity of the openings of the salivary ducts (cf. King, 1945; King and Glover, 1945). In marked contrast to lesions due to calculus alone, the epanutin disease was characterised, not by alternating hypertrophy and atrophy producing gingival recession, but by the formation of numerous lobular pedunculated growths springing from the gum external to and apparently some little distance from the sites of irritation by calculus.

Series 2 (Table II).—In order to determine whether or not the epanutin lesions were due to direct local action of the drug in the mouth, a further 16 ferrets were divided into two groups, one of which received epanutin in the food and the other intramuscularly (Figs. 5 and 6). Four more animals acted as controls. In this series also, an attempt was made to reduce the influence of inflammatory factors (calculus, food debris, etc.) by a 15-week period of bone administration before such local factors were allowed to come into play. The nature, progress and severity-distribution of the lesions of the 12 bone-deficient ferrets were identical, but the dietary supplements were usually associated with rather more advanced lesions. In comparison with Series 1 the preliminary period on bone, or perhaps the smaller intake of epanutin, appeared to have accentuated the lobular appearance of the growths, but the tooth crowns were less concealed. None of the 6 animals (2 controls) receiving bone throughout showed gingival abnormalities.

TABLE II (Series 2).—*Comparative Macroscopic Effects of Epanutin in the Food and Given Intramuscularly, with and without Dietary Bone. Age at Beginning, 8 weeks. Experimental Period, 43 weeks.*

Number of ferrets.	Experimental supplements.			Gum lesions observed.
	Epanutin.		Dietary bone.	
	In the food.*	Intramuscular.†		
6	48 mg. weekly	None	For first 15 weeks	Pedicles.
2	" "	"	Throughout	None.
6	None	48 mg. weekly	For first 15 weeks	Pedicles.
2	"	" "	Throughout	None.
2	"	None	For first 15 weeks	Inflammatory.
2	"	"	Throughout	None.

* 8 mg. epanutin daily from Monday to Saturday each week.
† 16 mg. epanutin thrice weekly (Monday, Wednesday, Friday).

and the 2 bone-deficient controls exhibited typical inflammatory lesions with alternating gingival hypertrophy and atrophy.

Series 3.—Eighteen ferrets were given 1–5 g. urea (B.D.H.) daily; 4 of the animals also received drinking water containing 2 per cent urea. All of the 10 bone-deficient ferrets were affected by gross gingival hypertrophy associated with calculus during experimental periods of 11–20 weeks. The later disease phases simulated the epanutin-type lesion (Fig. 8), but in the earlier stages the lobulations were less distinctive and evidences of inflammation were more prominent. In this series 6 animals receiving dietary bone remained free from visible gum disease and calculus and 2 others were in the process of cure by bone-gnawing when the experiment was ended.

Series 4 (Table III).—The parodontal changes resulting from dietary epanutin and urea (B.D.H.) were next compared under more appropriate experimental

TABLE III (Series 4).—*Comparative Macroscopic Effects of Epanutin and of Urea (B.D.H.) in the Food, with and without Periods of Dietary Bone. Age at Beginning, 12 weeks. Experimental Period, 22 weeks.*

Number of ferrets.	Experimental supplements.			Gum lesions observed.
	Epanutin.	Urea (B.D.H.).	Dietary bone.	
2	48 mg. weekly	None	For first 13 weeks	Pedicles.
2	None	12 mg. weekly	Ditto	,,
4	,,	,, ,,	None	,,
2	,,	,, ,,	Throughout	None.
2	,,	None	For first 13 weeks	Inflammatory.

conditions, the urea being given in amounts approximating those of the urea radicle in epanutin calculated on a molar basis (epanutin, 8 mg.; urea, 2 mg.). All of the bone-deficient ferrets given epanutin (2) or urea (6) developed gingival hyperplasia but, as suggested by the previous series, certain differences were apparent in the earlier stages. In the urea animals the characteristic strawberry-like lobulated growths were replaced by a more generalised swelling and congestion indicative of chronic inflammation, and later the gum margin was more readily lacerated by calculus and masticatory trauma (Fig. 7); these appearances were accentuated in the 2 ferrets which received no preliminary period of dietary bone. The 2 urea animals given bone throughout remained free from parodontal abnormalities.

Series 5.—Since it seemed possible that the lesions produced by urea might be associated with one or more of the known impurities in the commercial (B.D.H.) product, the latter was then compared as a dietary ingredient with pure urea prepared from human urine. Two pairs of ferrets were given 2 mg. daily of the crude and pure urea respectively for periods of 45 weeks in the absence of dietary bone. No obvious differences were found between the two groups, and in all 4 animals the gingival changes were similar to, but rather more advanced than, those illustrated in Fig. 7 owing to the longer experimental period.

Series 6.—Pairs of experimental skin excisions were made on two occasions in the backs of one control and one epanutin ferret. The results, in terms of rate of healing, were indecisive. Following the first operations, epithelialisation and exfoliation of scabs were complete in the control and epanutin animals by the 35th and 26th day respectively. Of the second wounds all showed healing by

the 24th day, but the epanutin scars were more raised and altogether more prominent during the first 5 weeks after operation. Even after 20 weeks characteristic stellate scarring persisted in the epanutin animal, while the scar had almost disappeared and new hair follicles were seen to be growing through the operation site of the control.

HISTOLOGICAL AND OTHER FINDINGS.

Epanutin.—The origin and progress of the mouth lesions are here considered in labio-palatal sections of gum, tooth and bone *in situ*. Despite the clinically normal appearance of the opanutin ferrets given dietary bone, proliferation of both subgingival and gingival epithelium is seen microscopically following dietary or intramuscular intake of the drug for 20 or more weeks (Fig. 9). The gingival epithelium is of special interest. In marked contrast to the early phases of gingivitis due to calculus, not only are the club-like proliferations remarkably uniform in thickness, but also the hyperplasia involves nearly the whole extent of the labial gum from gingival crest to cheek sulcus. The associated dilatation of the peripheral capillaries in the constricted inter-epithelial spaces undoubtedly leads to the mottled, strawberry-like appearance of the gum in the early clinical lesion (Fig. 1), this being confirmed by gingival capillaroscopy *in vivo* (King, 1944). Less obvious but more significant changes are also to be noted in the central parts of the gum corium, in the form of vaso-dilatation and perivascular proliferation of fibroblasts.

The later phases are seen in the epanutin animals deficient in dietary bone. The peculiar form of epithelial hyperplasia proceeds until many of the inter-epithelial projections of the corium are obliterated and the mass of epithelium begins to advance into the main body of the corium. But, a little distance from the site of calculus irritation, some of the original corium projections survive in the form of a leash of connective-tissue strands carrying with them fibroblasts and vessels from which a " pedicle " is to be derived. The site of pedicle formation is marked superficially by a slight elevation of the gum surface. Centrally the corium proper, though diminished in size by the invading gingival epithelium, again shows changes indicative of increasing density and vascularity ; progressive degeneration of the epithelial cells immediately coronal to the growing pedicle then causes a superficial groove by which separation of the pedicle from the parent tissue is facilitated (Fig. 10). In Fig. 11 a large pedicle is shown arising from the gum corium just below and external to the initial source of calculus irritation, and supported by two main connective-tissue columns which join one another at about the level of the original gum margin ; from this point the pedicle passes up on to the tooth crown where, in turn, its inner aspect is subject to injury by coronal calculus. Separation of the pedicle from the original gum is now well established and, at its inferior border, another surface groove is seen to mark the origin of a second pedicle. Around and between the principal fibrous supports of the main pedicle lie masses of hyperplastic but now rapidly degenerating gum epithelium which, at a later stage, will be largely replaced by new connective tissue. As each pedicle nears completion the ratio of its component corium to epithelium is abnormally increased, but the surviving epithelial cells may regain some of their normal activity and basophilic properties and the keratinous layer be re-established. In any case, however, the characteristic

pattern of the gingival epithelium is lost and the number of its cellular layers varies considerably from one area to another, depending upon the rate of growth of the underlying connective tissue. Towards the periphery the corium itself contains rapidly proliferating fibroblasts, plasma cells and capillaries reminiscent of granulation tissue, while its deeper portions consist of relatively avascular bundles of fibrous tissue. All of these changes, which may be repeated at different levels and on different aspects of the tooth-surrounding gum, result in the marked gingival enlargement typical of the epanutin disease. In the ferret there is basically a relative absence of leucocyte infiltration, but the process is later affected by inflammatory phenomena associated with invading calculus and masticatory trauma.

Outside the mouth no significant differences in gross post-mortem appearances of the tissues were found between the epanutin animals and their controls. As regards the eight skin wounds (2 ferrets), the experimental material was too scanty to permit definite conclusions from their gross or microscopic appearance, but the longer persistence of scarring in the epanutin animal and the more rapid healing with re-growth of hair in the control were confirmed histologically. The haemoglobin (Sahli) levels of 20 ferrets (*Series 2*) were remarkably constant, the percentage readings ranging from 95 to 114; these figures are within the normal range for the ferret. Some variations were apparent in the polymorph and lymphocyte counts, but their significance in this small series is at least doubtful.

Urea.—The histological appearances of ferrets' gingivae following *large* daily doses of urea in the diet and drinking water (*Series 3*) bear little resemblance to the epanutin material. It is true that there is again evidence of undue connective tissue as well as epithelial proliferation so that the entire tooth crowns may be hidden by the growths (Fig. 8). The general impression, however, is one of gross inflammatory hypertrophy due, as suggested by comparison with simple calculus lesions, to the concentrations of urea employed. Superficial areas of ulceration are common, and no doubt account, in part at least, for the observed extensive infiltration of the gum by polymorphs and other leucocytes. On the other hand, the animals receiving the *smaller* daily amounts of urea in the food (*Series 4* and *5*), whether commercial or purified, exhibit gingival lesions similar in some respects to those induced by epanutin including pedicle formation and connective-tissue proliferation. The gingival changes resulting from administration of large or small amounts of urea are again associated in site of origin and severity with calculus deposition and proximity of the salivary ducts. Details of the gingival lesions resulting from urea will be given when the results of further experiments have been evaluated. Finally, it should be mentioned that cavities have been found in the tooth roots of control, epanutin and urea animals (cf. King, 1944, 1949, 1951), but the impression has been gained that the incidence and extent of cavitation tend to be greater in the epanutin and urea series.

DISCUSSION.

Interest is here focused on the first reported production of so-called epanutin or dilantin hyperplasia of the gingivae in laboratory animals (ferrets), and its close similarity to lesions in human subjects under epanutin control. Other investigators, using small rodents, have had consistently negative animal results. For example, Shafer (1948) could find no evidence of gingival hyperplasia, gross

or microscopic, in any of 72 albino rats given epanutin at or considerably above the corresponding human therapeutic levels over periods of 13–14 weeks. In pilot experiments begun by the writer in 1947, 18 weanling rats and 10 adult hamsters were each given 1·6 mg. epanutin daily in the food for 29 and 15 weeks respectively. The main dietary ingredient was oatmeal or unground oats for the rats, while the hamsters were maintained on crushed "rat cubes." None of these animals developed gross epanutin-type lesions. In some, histological preparations revealed slight proliferation of the connective tissue of the gum corium and periodontal membrane, but no pedicle formation was observed. Further experiments on these animals are in progress, but already it seems clear that their parodontal tissues are less susceptible to the gross effects of epanutin than those of ferrets. In the latter species the epanutin lesions arise in the gingivae nearest to the openings of the main salivary ducts and to the tooth surfaces on which calculus first appears, but determination of the precise significance of gingival irritation is precluded by the present impossibility of eradicating or limiting tartar formation by methods other than bone-gnawing. The histological findings reported here are in general accord with those of Glickman and Lewitus (1941) and others dealing only with the human disease. Certain differences are apparent, but these may be related to the more rigid control of diet, calculus formation and epanutin administration in the animal experiments. There is certainly no evidence of species variation in susceptibility, and it is clear that both animal and human lesions are associated with intake of the drug and are totally unrelated to alleviation of convulsive disorders as such. It seems significant that in certain phases of the epanutin disease the enlarged gingivae are mainly composed of elements resembling those of granulation tissue and, since the gingivae are normally subject to persistent irritation of one kind or another, herein may lie the clue to their special susceptibility to proliferative stimuli.

Detailed discussion of the urea lesions in ferrets can serve no useful purpose here, since no precise connection with the epanutin disease has yet been established. It is, however, of interest that hyperplastic changes in the ferret's gum have resulted from daily doses of urea as low as 2 mg. per kg. body weight. Moreover, histologically, the lesions associated with administration of urea in small amounts can be differentiated from those due to calculus by the presence in the former and the relative absence in the latter of connective-tissue proliferation and pedicle formation. Although, then, the effects of urea may be partly or largely due to local irritation of the oral mucous membrane, some other explanation may be needed to account for proliferation of the gum corium.

SUMMARY.

The experimental production of proliferative lesions of the gingivae, associated with administration of epanutin and urea, is described in ferrets.

The epanutin disease is characterised initially by hyperplasia of the gum epithelium, differing in form and extent from that aroused by dental calculus and other local inflammatory agencies, together with proliferation of connective-tissue cells around the blood-vessels of the corium. At a later stage the corium expands at the expense of the epithelium, with the formation of numerous pedicles, the cellular, vascular and other components of which closely resemble those of granulation tissue. Gingival lesions due to urea administration are also asso-

ciated with connective-tissue proliferation and pedicle development, but differ from those of epanutin in being less defined and more affected by local inflammatory sequelae. Such connective-tissue proliferation is consistently absent in control ferrets allowed to develop gingivitis due to calculus alone, and this observation, reinforced by the production of excessive corium growth by epanutin given intramuscularly instead of orally, strongly suggests that, in the epanutin animals at least, the gingival growth stimulus is secondary to some primary disturbance elsewhere.

Comparison of the animal and human lesions resulting from epanutin administration reveals no essential difference between the gingival reactions of the two species.

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THE DEVELOPMENT OF INCLUSION BODIES IN THE CELLS OF THE RAT'S LIVER AFTER PARTIAL HEPATECTOMY.

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DURING an experimental investigation into factors related to the regenerative capacity of the rat's liver after partial hepatectomy, we noted the development of intracytoplasmic inclusions in the residual liver tissue during the first 24 hours after operation.

Although many types of inclusion bodies have been described in liver cells (Berg, 1914 ; Mallory, 1933 ; Li, 1936 ; Pappenheimer and Hawthorne, 1936 ; Belt, 1939 ; Weld, von Glahn and Mitchell, 1941 ; Rosin and Doljanski, 1944), there have been only sporadic references to such inclusions after partial hepatectomy (Gurd and Vars, 1949 ; Price and Laird, 1950 ; Aterman, 1952). Earlier workers (Fishback, 1929 ; Higgins and Anderson, 1931 ; Brues, Drury and Brues, 1936) made no mention of any inclusion bodies in the liver remnant. Our technique, however, had differed from theirs in that our animals were not starved before operation.

As will be seen, histochemical tests indicate that the inclusions consist largely of protein. This paper deals with the conditions of their production and discusses whether they indicate cellular damage or provide histological evidence of protein storage.

EXPERIMENTAL.

Stock albino rats of both sexes, weighing 150–250 g. were kept on a diet consisting of " Research " rat cubes supplemented with white bread and greens. Those rats designated as having fasted pre-operatively were given only water for a period of 24 hr. before operation.

Partial hepatectomy was carried out under ether anaesthesia after the method of Higgins and Anderson (1931), special care being taken to divide the ligaments of the median and left lateral lobes before ligation of their pedicles. By this method about two-thirds of the total liver mass is constantly removed. In 14 control rats killed immediately after partial hepatectomy we found the resected lobes to weigh 64·6 $\pm$ S.D. 2·7 per cent of the total liver.

The rate of regeneration was estimated by carrying out a two-thirds partial hepatectomy and killing rats at various intervals from 30 min. to 14 days later. The liver remnant was removed under ether anaesthesia, first ligating the portal vein and after 10 sec. the hepatic veins, and its weight was then plotted as a percentage of the estimated weight of the original whole liver as described by Brues *et al.* (1936). We found a rapid rate of regeneration similar to that recorded by previous workers (Higgins and Anderson, 1931 ; Brues *et al.*, 1936). The

remnant increased in weight from 35 per cent of the total liver mass immediately after partial hepatectomy to 42 per cent at the end of 24 hr., 61 per cent in 48 hr., 75 per cent in 72 hr., 83 per cent in 5 days, and then gradually reached complete restoration in about 14 days. The remnant, therefore, showed an exponential increase in its mass during the first 3 days. We found, by dry weights, as shown by Novikoff and Potter (1948), that this increase in weight is not due to an increase in water content.

The fixatives used were Helly, formol-saline and alcohol.

The Inclusion Bodies.

The inclusions first appear 10–30 min. after partial hepatectomy as very small intensely acidophilic intracytoplasmic bodies (never intranuclear), 3–5μ in diameter, more frequently in periportal than centrilobular cells (Fig. 1), and later increase in size to 15μ. They are not seen in the larger liver remnants examined 48 hr. after partial hepatectomy. The cells containing them appear to be healthy in so far as they contain a normal complement of cytoplasmic ribonucleic acid (Fig. 2) and apparently normal rod-shaped mitochondria (by phase contrast microscopy and on fixed tissue) (Fig. 3). The inclusion bodies give a strongly positive staining reaction for protein (acid dyes, Millon's reagent, phosphotungstic acid haematoxylin and Gram), and some but not all give a faint positive with Sudan black for the presence of non alcohol-soluble lipoid. Tests for haemoglobin (benzidine), neutral fat (Scharlach R on frozen sections), ribonucleic acid (pyronin, gallocyanin) and glycogen (Best's carmine) are all negative. They are present as refractile bodies in squashes made of unfixed unstained liver tissue.

The following experiments were carried out in order to determine factors which modify the development of the inclusions, and consisted in feeding and starving the rats prior to partial hepatectomy and in varying the amount of liver removed at operation.

The effect of feeding or starving rats for 24 hours before two-thirds partial hepatectomy.

As can be seen from Table I, pre-operative starvation strikingly lowered the incidence of inclusions. In a further small series, all the livers of 5 rats fed upon dextrose only before operation were also free of inclusions.

Comment: One of the effects of 4-2hr. starvation in rats is a rapid fall in the concentration of serum albumen (Cutting and Cutter, 1935). Our own findings are shown in Table II. It appeared to us possible that the absence of these protein

TABLE I—*Number of Rats showing Inclusions.*

Post-operative time.	Pre-operative feeding.		Total.	Pre-operative fasting.		Total.
	*0–Scanty.	†moderate–many		0–Scanty	Moderate–many	
½–4 hr.	0	17	17	14	3	17
4–24 hr.	4	12	16	9	0	9
	4	29	33	23	3	26

* 0-Scanty = less than 5 inclusions per whole section of liver lobe.
† Moderate-many = 5 or more inclusions per high power field.

TABLE II.—*Level of Blood Proteins in Fed and in 24-hour Fasting Rats in g. per 100 ml. Serum.*

	Fed rats.						Fasting rats.					
						Mean.						Mean.
Total	7·3	7·3	6·9	6·5	7·3	7·1	6·2	7·3	6·2	7·1	7·1	6·8
Albumen	3·9	3·9	3·5	3·5	3·8	3·7	2·5	2·7	2·4	2·7	2·8	2·6
Globulin	3·4	3·4	3·4	3·0	3·5	3·3	3·7	4·6	3·8	4·4	4·3	4·2
A/G	1·1	1·1	1·0	1·2	1·1	1·1	0·7	0·6	0·6	0·6	0·6	0·6

inclusions in starved rats and their presence in fed ones, might reflect a diminished capacity of the very small residual liver to deal normally with protein in the presence of a full plasma complement of albumen. This hypothesis could be tested by varying the relationship of residual liver and plasma albumen concentration.

One-third partial hepatectomy in fed rats with a normal concentration of serum albumen.

Five rats were subjected to this procedure and killed after 4 hr.: not one showed inclusions in the residual liver.

80–90 *per cent partial hepatectomy in starving rats with a lowered concentration of serum albumen.*

This was carried out by removing the right lateral lobe together with the median and left lateral lobes, leaving only the small caudate lobe. Of the 4 test rats in this experiment, all showed hepatic inclusions. All were killed 4 hr. after partial hepatectomy.

Comment: The results of these two experiments strongly support the hypothesis that there is for each level of plasma albumen (within the limits tested) a critical mass of liver tissue below which protein inclusions appear, above which they do not appear (Fig. 4).

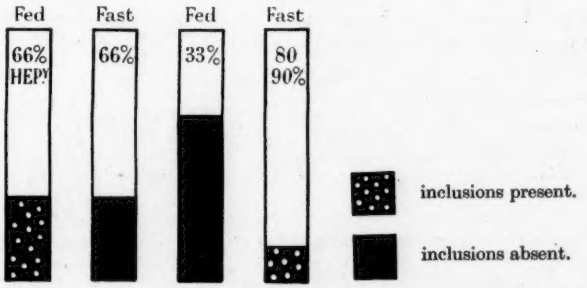


FIG. 4.—Diagram showing formation of inclusions in relation to liver mass and plasma protein level. Solid portion of each block represents residual liver mass, empty portion, the percentage of liver removed at partial hepatectomy.

Serum Protein.	Fed.	Fast.
Total	7·1	6·8
Alb.	3·7	2·6
Glob.	3·3	4·2
A/G	1·1	0·6

Differentiation from other inclusion bodies.

These inclusions are relatively easy to differentiate from most of the other inclusions described in the liver (Mallory, 1933 ; Pappenheimer and Hawthorne, 1936 ; Belt, 1939 ; Rosin and Doljanski, 1944).

One variety of inclusions occasionally seen by us was produced by accidental obstruction to the venous outflow of the residual lobes. These consisted of large cytoplasmic vacuoles (often indenting the nuclei), some of which contained varying concentrations of protein and were associated with focal intense congestion, necrosis or infarction (Fig. 5). They correspond exactly to the watery vacuoles described by Trowell (1946), who demonstrated that the conditions required for their production were anoxia combined with a normal or raised intrasinusoidal pressure.

Table III, which deals with rats not included in the previous charts, shows that the occurrence of vacuoles bears no relation to the animals' nutrition. In addition

TABLE III.—*Number of Rats showing Vacuoles after Accidental Vein Ligation.*

Post-operative time.	Pre-operative feeding		Total.	Pre-operative fasting.		Total.
	Absent.	Present.		Absent.	Present.	
4 hr.	0	2	2	0	2	2
4–24 hr.	0	5	5	0	1	1
	0	7	7	0	3	3

we were able to induce them in every one of 14 rats by deliberate ligation of the hepatic vein of the left lateral lobe. They then appeared in the obstructed lobe within 2 min. as large watery vacuoles which later became eosinophilic and some of which gave a positive stain for haemoglobin.

Comment : The morphology, time of onset, mode of production and staining reactions all differentiate "watery" vacuolation from the protein inclusions which we described previously ; this differentiation has, however, not been made in recent papers in which inclusions have been noted in residual liver lobes in rats (Gurd and Vars, 1949 ; Aterman, 1952).

EXPLANATION OF PLATES.

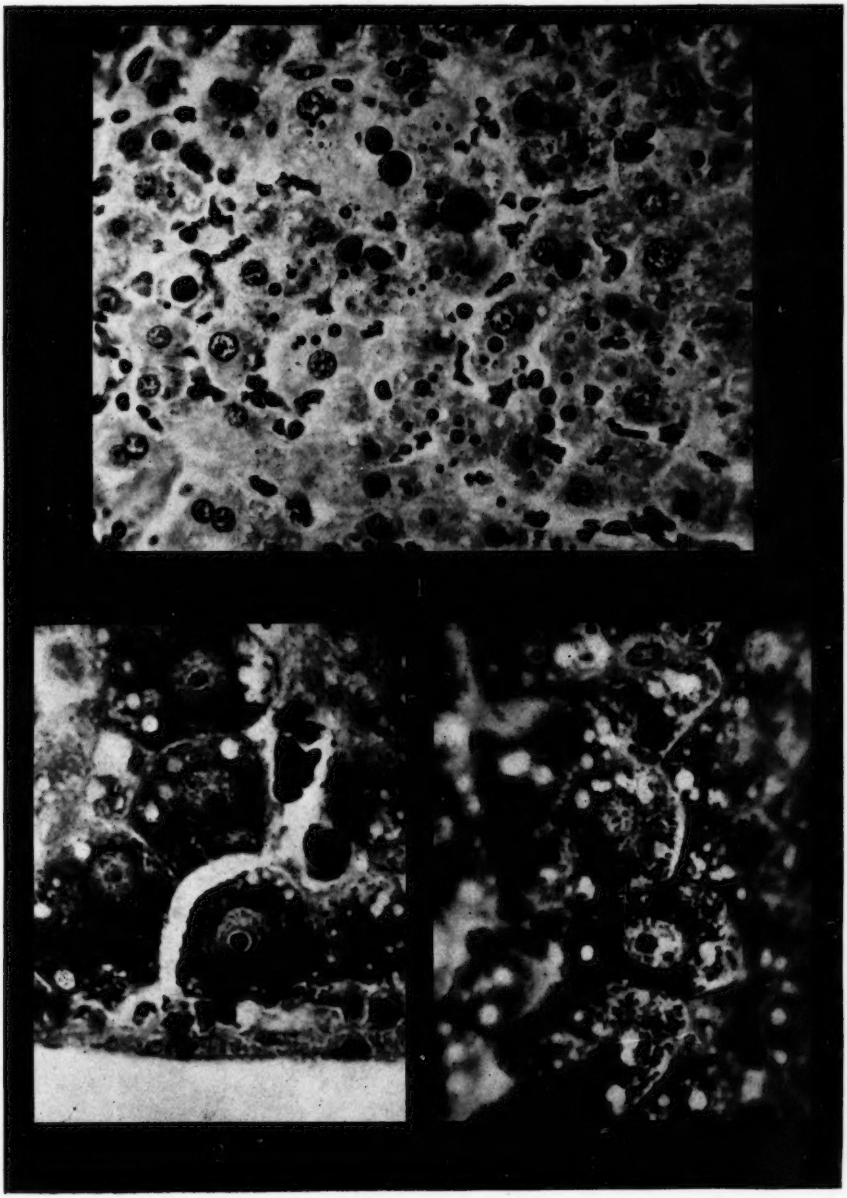
FIG. 1.—Regenerating liver of a feeding rat killed 4 hr. after a two-thirds partial hepatectomy, celestin blue, haemalum and phloxine, × 450 ; shows the general character and variation in size of inclusion bodies, all of which are strongly phloxinophil.

FIG. 2.—Liver from rat of same series as Fig. 1, Orange G, pyronin methyl green mixture (Trevan and Sharrock, 1951), × 1150 ; shows a cell containing an inclusion. There is abundant pyroninophilic material in the cytoplasm.

FIG. 3.—Liver from rat of same series as Figs. 1 and 2, Altmann's aniline acid fuchsin—methyl green (Bensley), × 1000 ; shows normal rod-like mitochondria in an inclusion-bearing cell.

Fig. 5.—Liver from rat killed 4 hr. after partial hepatectomy and accidental hepatic vein ligation, celestin blue, haemalum and phloxine, × 450 ; shows vacuoles, some of which indent the nuclei. The contents of the vacuoles vary from weekly to intensely phloxinophil.

FIG. 6.—Feeding rat killed 4 hr. after a two-thirds partial hepatectomy and synchronous intravenous injection of 2 ml. 1 per cent Evans blue. Unstained formol fixed frozen section, × 130. The inclusions are all heavily stained by the Evans blue ; a little dye has been taken up by the Kupffer cells.



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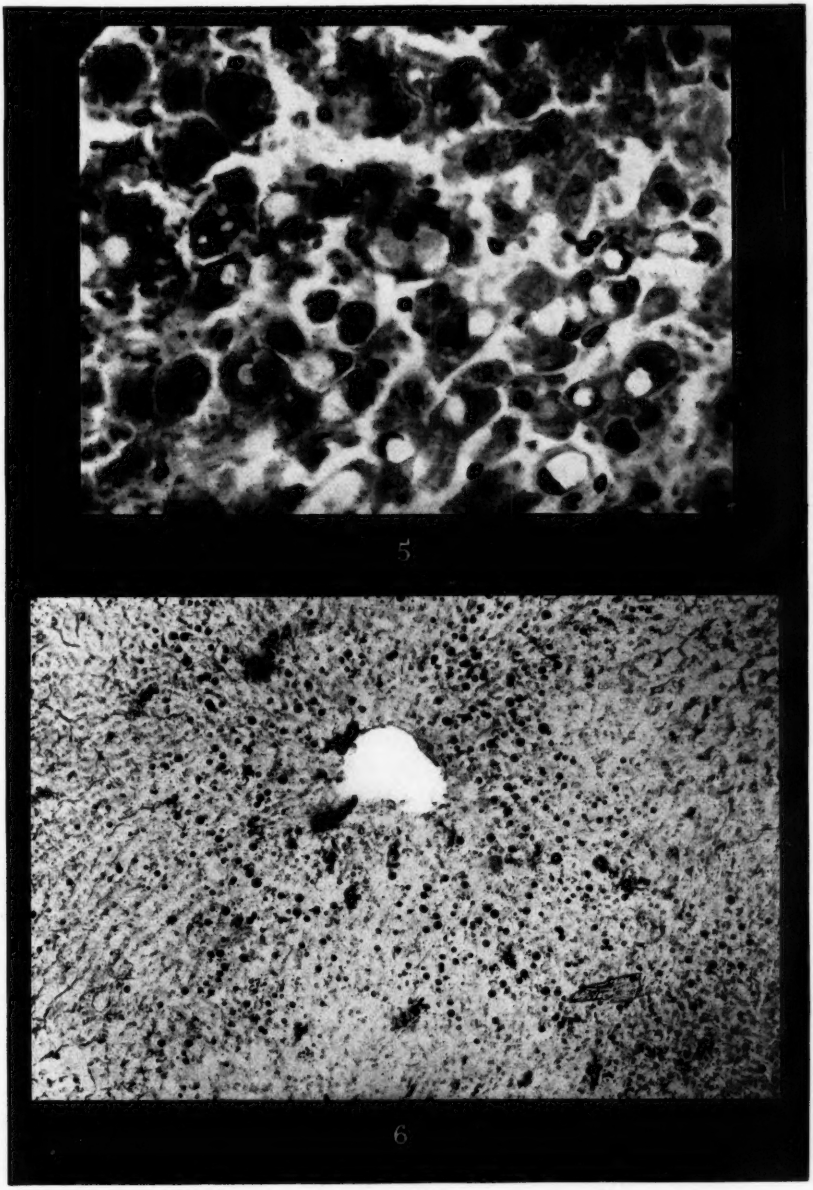

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Uptake of T. 1824 (*Evans Blue*) *by these inclusions from the circulation.*

In an attempt to identify one possible source of the protein inclusions, the plasma albumen was labelled with T.1824 in a very weak dilution of 1 molecule of dye to 20 of plasma albumen. In this degree of dilution, Rawson (1943) and Allen and Orahovats (1951) suggest that the dye exists entirely as a dye-albumen complex. After injecting 1 ml. of very dilute dye intravenously at the time of hepatectomy and examining a frozen unstained section of liver from a rat killed 4 hr. later, occasional inclusions were seen to show a faint dye staining. If, however, an excessive concentration of Evans Blue dye is used (5 molecules dye to 1 of albumen), so that there is free circulating dye, then every inclusion is very heavily stained (Fig. 6), with no staining of the rest of the cytoplasm. Watery vacuoles do not take up Evans Blue in this fashion.

Comment: The fact that freely circulating Evans Blue is taken up so avidly by the inclusion bodies, furnishes additional evidence of their protein nature, and the trace of staining produced after injection of the very weak solution suggests that they are made up in part of plasma albumen.

DISCUSSION.

The predominantly protein nature of the inclusion bodies has been demonstrated. Their slight affinity for lipoid stains is regarded as due to the presence of a lipoid derivative of the cytoplasm at the zone of contact between inclusion body and cell cytoplasm. Our histological findings have shown no evidence of any damage to the cells bearing these inclusions. It is possible that they may be of significance in the protein metabolism of the liver cell.

Madden and Whipple (1940) and Whipple (1948) have shown that the production, storage and loss of body protein are in a balanced state, and they have produced evidence that plasma proteins are in dynamic equilibrium with tissue proteins. It has been demonstrated that the liver is a source of plasma proteins by Tarver and Reinhardt (1947) and Miller, Bly, Watson and Bale (1951). Part of the protein in the liver is labile, as much as 20 per cent being lost from the rat's liver after 1–2 days' starvation (Addis, Poo and Lew, 1936*a*, *b*). Dietary nitrogenous compounds have been shown to be rapidly incorporated in the plasma protein (Schoenheimer, Ratner, Rittenberg and Heidelberger, 1942; Friedberg, Tarver and Greenberg, 1948), and, on the other hand, intravenously injected ^{15}N-labelled plasma protein is incorporated rapidly in tissue protein and only slowly metabolised (Fink *et al.*, 1944). Shemin and Rittenberg (1944), also on the basis of isotopic studies, have postulated that the flow of protein constituents takes place in both directions between liver and plasma. It is abundantly clear, therefore, that the liver plays a key part in the maintenance of the protein balance of the body as both a source and storage site and possibly as a regulator *via* the plasma.

The inclusion bodies develop at a critical relationship of liver mass and plasma albumen level. It would appear that after partial hepatectomy the small liver remnant in the presence of a relatively large protein pool either holds on to the protein material passing through it or accumulates excess protein. The normal liver is thought to form plasma protein and pass it into the circulation at a faster rate than it forms its own cellular protein (Tarver and Morse, 1948), but in the experimental conditions imposed in this investigation this process is probably

disturbed. Excessive accumulation of protein is not a striking feature in normal protein metabolism and excess nitrogen is easily eliminated (Folin, 1905). However, in this series of experiments the sudden reduction of mass of functioning liver tissue, associated with a relatively grossly increased portal blood supply in an animal with a normal protein pool, apparently imposed a stimulus for storage of protein in the liver.

An apparent histological demonstration of stored hepatic intracellular protein has been described previously (Berg, 1914 ; Li, 1936), but the material described, with its variations following feeding and fasting, has been clearly identified as containing ribonucleic acid (Deane, 1946 ; Davidson and Waymouth, 1946 ; Stowell, 1948 ; Lagerstedt, 1949). The inclusion bodies we describe have been differentiated from ribonucleic acid-containing granules.

The inclusions are dealt with by the liver cell in 24–48 hours, after which they do not develop. It seems that at such a period after partial hepatectomy there is sufficient liver mass available to metabolise protein in the more usual way. The mechanism which determines whether protein material will be stored in the manner described is difficult to analyse. The relationship between the two levels (plasma albumen and functioning liver mass) appears to be one of the factors which determine the rate or direction of flow of proteins between liver cell and plasma.

With regard to the regeneration of the liver, it is conceivable that the inclusions might not only provide extra material for growth, but that their presence might by itself be a partial, though not essential, direct stimulus to hyperplasia and division of the liver cells.

SUMMARY.

Cytoplasmic inclusion bodies of predominantly protein nature have been demonstrated in the liver remnant after two-thirds partial hepatectomy in feeding rats. They are not produced in starving rats or in rats fed only upon dextrose.

These inclusion bodies are regarded as histological evidence of protein storage.

The relationship between the amount of functioning liver tissue and the level of albumen in the plasma protein appears to determine the rate and direction of the flow of protein constituents between liver and plasma.

We wish to thank the Department of Chemical Pathology of the Postgraduate Medical School for carrying out the serum protein determinations, Mr. J. G. Griffin and Mr. L. J. Wright for the sections, and Mr. E. V. Willmott for the photomicrographs.

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AVAILABILITY OF WATER IN ANTIGEN-ANTIBODY PRECIPITATES FOR SOLUTION OF LARGE MOLECULES.

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THE combination of antibody molecules with antigen molecules depends on the attraction between specific receptor sites on the surfaces of the antibody molecules and specific determinant groups on the surfaces of the antibody molecules. A receptor site of an antibody molecule must be in contact with a determinant group of an antigen molecule with which it is combined ; the molecules must be correctly oriented in order that the receptor may fit the determinant group. The antibody molecules are not packed indiscriminately round an antigen molecule. According to the lattice theory, each antigen molecule combines with several antibody molecules and each antibody molecule with two or possibly more antigen molecules ; a large three-dimensional network is built up. It might be expected that there would be spaces in this network in which there would be room for water and other small molecules, but not for molecules whose size was of the order of that of the antigen. Even if each molecule of antibody is combined with one antigen molecule only, there may be gaps between the antibody molecules that will not accommodate other large molecules. But, according to the occlusion theory (Boyd, 1942) of the formation of precipitates by antigens and antibodies, antibody molecules are in close contact and there are no such gaps between them. We have measured the water in antigen-antibody precipitates, that is not available for haemoglobin, by comparing the concentrations of haemoglobin in the compact precipitate and the supernatant fluid.

MATERIALS.

Ovalbumin was crystallised from egg white by the method of Hopkins and recrystallised twice. Type I pneumococcal polysaccharide (S_1) was prepared by Mr. R. G. S. Johns by the method of Heidelberger, Kendall and Scherp (1936).

Rabbit antisera against ovalbumin were prepared by immunizing rabbits by Proom's (1943) method. Gamma-globulin was prepared from these antisera by the method of Kekwick (1940) slightly modified (Marrack, Hoch and Johns, 1951) to remove small amounts of β-globulin and get a clear solution ; after the pH of the solution had been adjusted to about 7·2 the solution was dialysed against 0·9 per cent NaCl solution.

Horse anti-pneumococcal serum, Type I, was provided by the Lister Institute ; it was made in 1939 and contained 0·5 per cent phenol. The antibodies in this serum were concentrated by dialysis against a mixture of 78 ml. of 0·066 M

Na_2HPO_4 and 143 ml. of 0·066 M KH_2PO_4 diluted to 1 litre. The globulin was dissolved in 0·9 per cent NaCl solution and neutralised. Rabbit anti-pneumococcal serum, Type I, was made by Mulford Laboratories; the euglobulin was precipitated by the method used for concentrating the antibody of horse serum and the pseudoglobulin, which contained the antibody, was then precipitated by dialysis against 18 per cent Na_2SO_4; the precipitate was dissolved and dialysed against 0·9 per cent NaCl solution.

A sample of crystalline human haemoglobin was given us by Dr. Perutz; crystalline human and horse haemoglobin were made by Drabkin's (1949) method, dissolved in 0·9 per cent NaCl solution and filtered through a Seitz filter before use.

METHODS.

A solution of antibody was mixed, at room temperature, with a solution of haemoglobin, making the final concentration of haemoglobin slightly over 1 per cent; antigen was then added gradually, with stirring. The mixture was left at room temperature until floccules had appeared and then in a refrigerator at 4° overnight; it was then centrifuged at 3000 r.p.m. for 1 hr. in a weighed stoppered centrifuge tube. The supernatant fluid was then removed with a pipette and the tube weighed again. The precipitate was dissolved and the tube with the contained solution was weighed. In Experiments 1 and 2, 0·1 N NH_4OH was used to dissolve the precipitates. Haemoglobin was estimated in weighed aliquots of this solution and of the supernatant fluid by King's (1946*a*) cyanmethaemoglobin method; it was found that the presence of relatively large quantities of γ-globulin did not affect the optical density. The precipitates formed by ovalbumin did not dissolve completely in 0·1 N NH_4OH; these precipitates were therefore dissolved in 0·1 N NaOH. Attempts were made to measure the haemoglobin by King's (1946*b*) haematin method; later it was found that this method was unsatisfactory when relatively large amounts of other protein were present. In Experiments 3–6 the precipitate was dissolved in 0·1 N NaOH and the haemoglobin was estimated by the pyridine-chromogen method of Rimington (1942) slightly modified.

The light absorption was measured in a Hilger Uvispek spectrophotometer at wave-length 422 mμ. Absorption at this wave-length is about 5 times that at 555 mμ. At concentrations of haemoglobin above 0·02 per cent (final dilution) there was a small positive deviation from Beer's law at 555 mμ., and a negative deviation at 422 mμ. Measurements were therefore made on solutions containing less than 0·2 mg. haemoglobin per ml. It was found that the light absorption is increased when relatively large amounts of γ-globulin are present. As good agreement with Beer's law was found with haemoglobin in presence of a constant excess of γ-globulin as with haemoglobin alone. A correction was therefore made to allow for this effect of γ-globulin; this did not in any case amount to more than 7 per cent.

Weighed aliquots of the solutions of the precipitate and the supernatant fluids were dried *in vacuo* over silica gel to constant weight. The water was obtained by difference; when 0·1 N NaOH was used to dissolve the precipitates, allowance was made for the few mg. of Na added. The concentration of haemoglobin in the water of the precipitate could then be compared with the concentration in the water of the supernatant fluid.

Example. Experiment 1; antigen S_1.

Weight of wet precipitate	0·9951 g.
Weight of solution of precipitate in 0·1 N NaOH	4·3925 g.
Dry weight in 2·3965 g. of solution of precipitate	0·0715 g.
Dry weight in total wet precipitate	0·131 g.
Water in total wet precipitate	0·864 g.
Haemoglobin in 0·998 g. of solution of precipitate	1·86 mg.
Haemoglobin in total wet precipitate	8·25 mg.
Haemoglobin per 100 g. precipitate water	0·95 g.
Haemoglobin per 100 g. supernatant fluid	1·40 g.
Haemoglobin per 100 g. water of supernatant fluid	1·44 g.

The concentration of haemoglobin per 100 g. water is, therefore, less in the precipitate than in the supernatant fluid. At the concentration found in the supernatant fluid (1·44 g. haemoglobin per 100 g. water) the haemoglobin in the precipitate (8·25 mg.) would be contained in 0·57 g. water. Therefore, 0·864—0·57 = 0·29 g. precipitate water was not available for solution of haemoglobin. The protein of the specific precipitate was 0·10 g.; so that 2·9 g. water per 1 g. specific precipitate protein were not available for solution.

RESULTS.

In all the experiments, except No. 6, the dry matter, after prolonged centrifuging, was not more than 12 per cent of the total wet precipitate; in Experiment 6 the dry matter amounted to 23 per cent. of the wet precipitate. The non-available water (Table I) was over 2 g. per 1 g. specific precipitate protein when the antigen

TABLE I.—*Non-Available Water in Antigen-Antibody Precipitates.*

Expt. No.	Antigen and antibody.	Ratio of antibody to antigen.	Specific precipitate protein. (mg.)	Non-available water. (g.)	Non-available water per 1 g. specific precipitate protein. (g.)
1	S_1; horse antibody	Equivalent	100	0·29	2·9
2	Ditto	,,	100	0·21	2·1
3	,,	7 times equivalent	65	0·205	3·1
4	Ovalbumin; rabbit antibody	110 mg. antibody to 10 mg. antigen	120	0·154	1·3
5	Ditto	63·7 mg. antibody to 3·3 mg. antigen	67	0·090	1·35
6	S_1; rabbit antibody	Equivalent	60	0·139	2·3

was S_1 (Experiments 1, 2, 3 and 6), and over 1·3 g. per 1 g. specific precipitate protein when the antigen was ovalbumin. This difference cannot be attributed to differences in the sizes of the antibody molecules, as similar results were obtained with horse and rabbit anti-pneumococcal antibodies. The difference may be due to the structure of the antigen; the precipitates formed by the polysaccharides with both rabbit and horse antibodies are gelatinous, while the precipitates formed by protein antigens are granular.

The distribution of glucose, estimated by Somogyi's (1945) method, and of Cl, estimated by the method used by Marrack and Hewitt (1927), were also measured (Table II). There is no evidence of appreciable amounts of water that are not

TABLE II.—*Concentration of Glucose and Cl in Antigen-Antibody Precipitates and in the Supernatant Fluid.*

Antigen and antibody.	Ratio of antibody to antigen.	Precipitate.		Supernatant.	
		Protein.	Glucose.	Protein.	Glucose.
		(g. per 100 g. water.)			
S_1; horse antibody . Ovalbumin; rabbit antibody	Equivalent .	13·5 .	1·55 .	0·54 .	1·57
	„ .	13 .	1·045 .	0·9 .	1·05
		Protein.	Cl.	Protein.	Cl.
		(g. per 100 g. water.)			
S_1; horse antibody . Ovalbumin; rabbit antibody	Equivalent .	13·5 .	0·555 .	1·8 .	0·545
	„ .	9·4 .	0·52 .	2·1 .	0·52

available for solution of these smaller molecules. It appears, therefore, that there is a considerable amount of water in the complexes formed with antibody by the polysaccharide into which relatively small molecules can penetrate but from which molecules of the size of haemoglobin are excluded; and that there is a considerably smaller amount of such non-available water in the complexes formed by ovalbumin. It does not appear that the antibody molecules, that form the greater part of the precipitates, are closely packed, so that their polar groups are not free to attract water.

SUMMARY.

The water that is not available for solution of haemoglobin in the precipitates formed by Type I pneumococcal polysaccharide and ovalbumin with the corresponding antibodies has been measured.

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Our thanks are due to Dr. M. F. Perutz for a gift of crystalline haemoglobin.

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THE EFFECT OF CORTISONE ON EXPERIMENTAL INTRAPERITONEAL SILICOTIC NODULES.

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WE have shown in a previous paper (Magarey and Gough, 1952) that cortisone in large doses reduces to some extent the formation of fibrous tissue provoked by finely powdered quartz in the peritoneal cavity of rabbits.

The experiments described in the present paper were undertaken to determine whether cortisone has any effect upon the fibrous connective tissue of established silicotic nodules.

METHODS.

Silicotic nodules were produced in the peritoneal cavity of rabbits by injecting —without anaesthetic—2 ml. of a 10 per cent suspension of quartz powder in normal saline. The quartz powder was similar to that used in our previous experiments, and consisted of a Belgian sand (Snowit II) ground to a particle size of less than 2μ diameter. The saline suspension was autoclaved and shaken with glass beads in a Kahn shaker for an hour immediately before use to break up any aggregations of particles.

After a period of 70 days the animals were anaesthetised and the peritoneum examined by means of a rather wide-bore (about 10 mm.) human urethrascope inserted through a lateral abdominal incision. Any well-developed silicotic nodules on the peritoneal surface of the anterior abdominal wall were immediately recorded by coloured drawings. Six animals were selected and matched in pairs according to the pictures of the nodules.

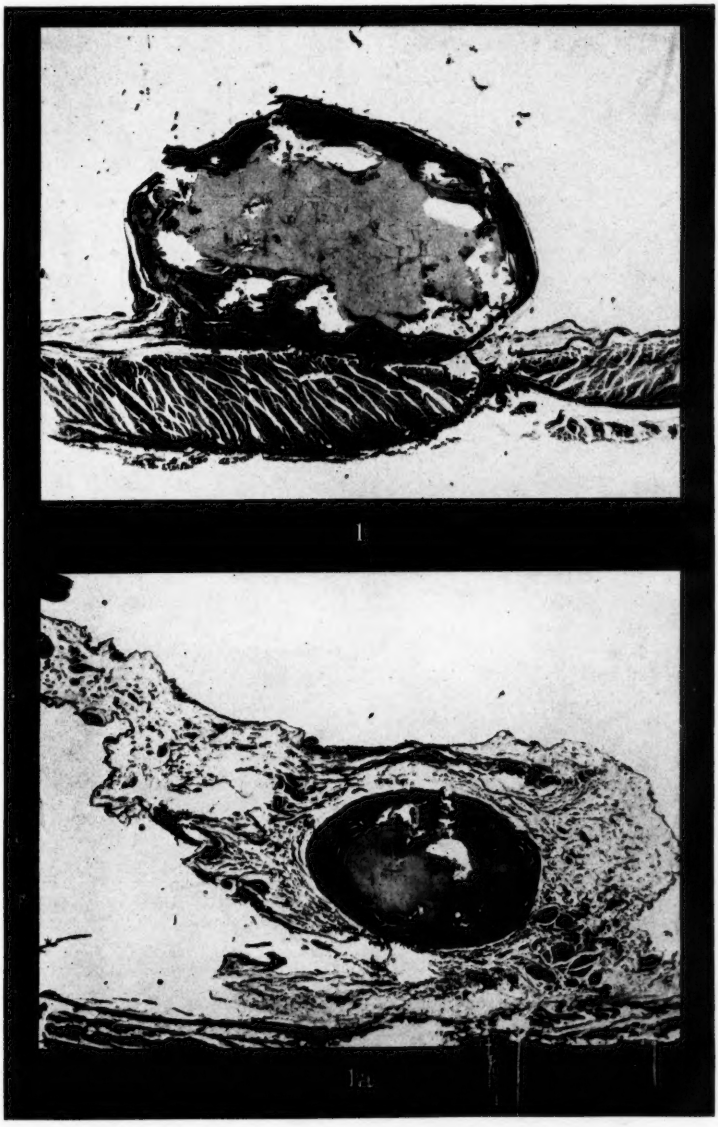
DESCRIPTION OF PLATES.

FIG. 1.—Silicotic nodule from control animal. The central collection of quartz powder is surrounded by a dense collagenous capsule. Haemalum and Van Gieson, × 9.

FIG. 1*a*.—Silicotic nodule from a cortisone-treated animal. The collagenous capsule is surrounded by new delicate, rather vascular connective tissue. Haemalum and Van Gieson, × 9.

FIG. 2.—From control animal. On the left can be seen a broad cellular fringe outside the dense layer of compact collagen. Haemalum and Van Gieson, × 80.

FIG. 2*a*.—From the cortisone-treated animal. Instead of the cellular fringe there is a diffuse delicate connective-tissue reaction consisting of loosely knit fibres and thin-walled blood vessels. Haemalum and Van Gieson. × 80.



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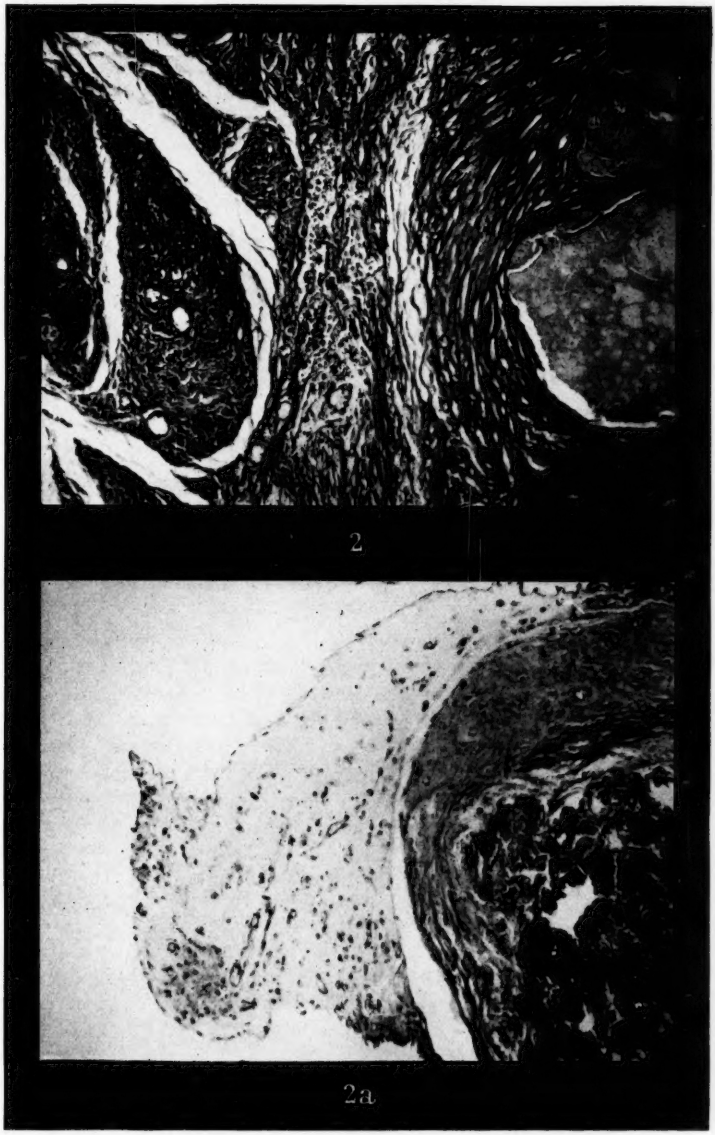

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Magarey and Gough.

After allowing two weeks for the complete healing of the laparotomy wound, one of each pair of rabbits was given 20 mg. cortisone acetate daily, the other being kept as a control. At intervals of 22, 36 and 84 days after the commencement of the cortisone intraperitoneal examination of a pair was made, the lesions re-drawn and the animals then killed.

RESULTS.

When the drawings of the nodules before and after cortisone were compared no change could be seen. Likewise there was no essential macroscopic difference between the nodules in the cortisone-treated rabbits and those in the controls.

Histologically in the control animals the nodules consisted of a central accumulation of quartz surrounded by a dense layer of compact collagen (Fig. 1), and outside this a cellular fringe in which the presence of many fibroblasts suggested that active fibrosis was still progressing (Fig. 2).

In the cortisone-treated animals the nodules were similar (Fig. 1*a*) except that there was no cellular fringe (Fig. 2*a*), but a different form of reaction in which a diffuse delicate connective tissue, consisting of loosely knit fibres and thin-walled blood-vessels, surrounded the silicotic nodules. This redundant vascular tissue corresponds to that seen in similar experiments previously described (Magarey and Gough), and probably also to the diffuse fibrosis noted by Curran (1952), who reported on the effects of silica in the peritoneal cavities of mice. It appears to be a special effect of cortisone in the presence of silica.

DISCUSSION.

Following our previous finding that cortisone could modify the formation of fibrous tissue provoked by quartz powder, we have investigated whether it could also cause any change in silicotic fibrous tissue already formed.

This problem assumes some importance on two counts: Firstly cortisone is widely reported as having a beneficial effect on certain chronic conditions characterised by long-standing fibrosis such as scleroderma and keloid scarring. Secondly there has been a report of appreciable clinical improvement in a case of pulmonary silicosis treated with adrenocorticotrophic hormone (Kennedy *et al.*, 1951).

Curran showed that cortisone had no effect upon established intraperitoneal silicotic lesions in mice. We have likewise been unable to show any reduction of collagen even with large doses of cortisone on established intraperitoneal silicotic nodules in rabbits. The absence of a cellular outer fringe in the nodules in cortisone-treated animals, however, suggests that during the administration of the cortisone there was the effect that further fibroblastic proliferation was retarded.

It would therefore seem justifiable to suggest that cortisone would not be expected to have any lasting beneficial effect upon the collagen in human silicosis, although it might well arrest progression of the lesions during the period of administration.

SUMMARY.

Intraperitoneal silicotic nodules were established in rabbits and the effect of cortisone on these lesions determined. The pre-existing dense collagenous tissue remained unchanged. There was however retardation of further concentric

fibrosis during the administration of the cortisone, its place being taken by a diffuse connective tissue and vascular proliferation.

We are very grateful to Dr. G. Nagelschmidt for the quartz powder, and to Mr. J. P. Napper, Mr. G. R. Armstrong and Miss June Williams for technical assistance.

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